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LXVII. THE DETERMINATION OF THE TERTIARY DISSOCIATION CONSTANT OF PHOSPHORIC ACID.

By ISAAC NEWTON KUGELMASS.

From the Department of Pediatric Research, The Fifth Avenue Hospital, New York.

(*Received March 11th, 1929.*)

Quantitative studies of the mechanism of calcification require a knowledge of the physico-chemical constants of the blood equilibria involved in the process [Kugelmass, 1924; Kugelmass and Shohl, 1924].

The values for the tertiary dissociation constant of phosphoric acid available at present are at variance. Abbott and Bray [1909] found K_3 to be $3{\cdot}6 \times 10^{-13}$ at 18° for a total concentration of 0·026 *M* by means of conductivity and distribution ratios of ammonia between chloroform and solutions of sodium ammonium phosphate. This value seems incompatible with the apparent dissociation constant p_{K_3}, from the dissociation curve for the phosphates, which is of a higher order of magnitude.

The tertiary dissociation constant of phosphoric acid was determined from a study of the aqueous systems of the tertiary phosphates at equilibrium from the standpoint of hydrolysis.

The neutralisation of orthophosphoric acid by strong alkali has been studied by means of thermochemical, electrometrical, and conductance measurements. Thomsen has shown that the small heat effects that attend the addition of the third equivalent of base indicate that the third equivalent of hydrogen of the acid is far from completely replaced when the proportions of acid and base are those corresponding to the salt Na_3PO_4. Berthelot found that the decrease of conductance during neutralisation may be represented by a straight line up to the point at which the first hydrogen of the acid is replaced and by another straight line different in direction from the first up to the point corresponding to the replacement of the second hydrogen. Beyond this point further addition of alkali causes a change in the direction of the curve, which can no longer be represented by a straight line. Hence, the first and second equivalents of hydrogen in orthophosphoric acid are almost quantitatively replaced but the third equivalent is only gradually neutralised as the proportion of base increases. These results clearly indicate that the tertiary phosphate is hydrolysed to a considerable extent.

THEORETICAL.

Derivation of equations. The tertiary dissociation constant may be evaluated from the degrees of hydrolysis of the tertiary phosphates. The hydrolysis occurs progressively in several stages, to each of which correspond definite equilibria represented by the following stoichiometric equations:

$$\begin{aligned} Na_3PO_4 + H_2O &\rightleftharpoons Na_2HPO_4 + NaOH \\ Na_2HPO_4 + H_2O &\rightleftharpoons NaH_2PO_4 + NaOH \\ NaH_2PO_4 + H_2O &\rightleftharpoons H_3PO_4 + NaOH \end{aligned} \qquad \text{.....(1).}$$

The tertiary phosphate hydrolyses to a great extent, the secondary and primary salts inappreciably, as will be evident from the data below. Such an aqueous system probably also contains the intermediate ions, $Na_2PO_4^-$ and $NaPO_4^=$, but we know very little about their concentrations or activities. Hence, the first stage hydrolysis (equation (1)) may be considered as the essential equilibrium.

The mechanism of equation (1) may be expressed by the ionic equilibrium:

$$PO_4^{\equiv} + H_2O \rightleftharpoons HPO_4^{=} + OH^-.$$

Applying the law of mass action,

$$\frac{[HPO_4^=][OH^-]}{[PO_4^{\equiv}]} = K_h \qquad \text{.....(2),}$$

where the brackets include the ion activities; K_h is the "activity" hydrolysis constant.

The degree of hydrolysis, h, is by definition,

$$h = \frac{[HPO_4^=]}{\gamma_S C} = \frac{[OH^-]}{\gamma_B C} \qquad \text{.....(3)}$$

and

$$1 - h = \frac{[PO_4^{\equiv}]}{\gamma_T C},$$

where γ_B, γ_S, γ_T are the activity coefficients of hydroxide, secondary and tertiary phosphates of sodium or potassium respectively and C is the total molar concentration of all phosphates. Substituting the values of OH^-, $HPO_4^=$ and $PO_4^{\equiv}$ from these relations in equation (2), we have

$$\frac{h^2 \gamma_B \gamma_S C^2}{(1-h)\gamma_T} = K_h \qquad \text{.....(4).}$$

Also, the condition obtains that

$$[H^+][OH^-] = K_w.$$

Dividing this by equation (2),

$$\frac{[H^+][PO_4^{\equiv}]}{[HPO_4^=]} = \frac{K_w}{K_h} = K_a \qquad \text{.....(5),}$$

where K_a is the tertiary ion activity or true constant.

The apparent dissociation constant may also be expressed in terms of the degree of hydrolysis of the tertiary salt

$$K_3' = \frac{[H^+][Na_3PO_4]}{Na_2HPO_4} \qquad \text{.....(6).}$$

Replacing for the secondary and tertiary salt concentrations their equivalents from the above relationships

$$K_3' = [H^+]\left(\frac{1-h}{h}\right) \quad \ldots\ldots(7).$$

Expressed in logarithmic form

$$p_H = p_{K_{a'}} + \log\frac{1-h}{h} \quad \ldots\ldots(8).$$

Experimental.

Method of procedure. Purely prepared sodium and potassium phosphates were made up to definite concentrations with conductivity water at 20° and kept in pyrex flasks. The hydrion concentrations of these solutions were measured potentiometrically in a thermostat maintained at 20° and 38° $\pm$ 0·02° respectively. The gas chain consisted of Pt—H_2—solution x—saturated KCl—HgCl—Hg. The hydrion potentials measured for these solutions at varying time intervals showed that the hydrolytic equilibria are established immediately on preparation of the solution.

The hydrion concentration measurements were standardised daily against 0·1 *N* HCl prepared from constant boiling acid. The p_H was 1·085 at 20° and 1·09 at 38°, extrapolated from the activity coefficients determined by Noyes and Ellis [1917]. The calculated E.M.F. values of the entire system against the acid standard were 0·3119 at 20° and 0·3022 volt at 38°. The average divergence in terms of p_H was 0·003. The observed voltages were corrected to one atmosphere of hydrogen.

Preparation and analysis of materials. The tertiary phosphates were recrystallised twice from distilled water free from CO_2 and finally again by the addition of an equal volume of 95 % alcohol and then cooling in ice water. Two liquid phases were formed upon addition of alcohol, crystallisation proceeded at the interface while the upper phase gradually disappeared. The upper layer in the closed system prevented the absorption of CO_2 which otherwise would have tended to form the secondary phosphate and carbonate. The $Na_3PO_4.12H_2O$ crystals are trigonal and the $K_3PO_4.2H_2O$ are needle-like. The crystals were filtered on a Büchner funnel with suction and washed several times with alcohol. They were dried on a glass plate and finally pulverised. No attempt was made to prepare constant hydrates but the crystals were dried to remove free moisture and analysed for water of hydration. The water content was determined by first weighing a sample of the material in a platinum crucible and then heating in the electric furnace until constant weight was obtained.

The dodecahydrated trisodium phosphate crystals were tested by determining the transition temperature, 73·4°, which Richards proposed as a fixed point in thermometry. Analysis of the phosphates gave the following results.

Percentage composition of the phosphates.

Salt	Water determined	P	
		Determined	Calculated
K_2HPO_4	27·41	13·82	13·86
Na_2HPO_4	45·65	12·70	12·77
K_3PO_4	34·84	9·50	9·47
Na_3PO_4	44·74	10·45	10·49

Experimental and calculated data.

$[H^+]$ is the activity of the hydrion taken as that determined by the hydrogen electrode.

γ_B, γ_S and γ_T are the activity coefficients of the hydroxide, secondary and tertiary phosphates, respectively of sodium or potassium. They are the thermodynamic degrees of dissociation obtained by dividing the mean activity of the ions or their effective concentrations by the molarity of the salt. The coefficients given were read from curves plotted from Lewis and Randall's data [1923] for uni-, bi- and ter-valent anions at the calculated ionic strengths. The validity of this procedure follows especially from recent work which indicates that the ratio of the activity coefficient of an ion of one valency to that of another is constant at a given ionic strength.

Table I. *The tertiary dissociation constant of phosphoric acid.*

Calculated from the hydrolysis of Na_3PO_4 at 20°.

C	H^+	OH^-	μ	γ_B	γ_S	γ_T	h	K_h	$K_a.10^{12}$	$K_a'.10^{-12}$
0·0250	$0·907 \times 10^{-12}$	$9·48 \times 10^{-3}$	0·126	0·79	0·23	0·18	0·480	$1·12 \times 10^{-2}$	0·77	0·98
0·0125	1·418 „	6·03 „	0·065	0·84	0·33	0·26	0·574	1·03 „	0·83	1·06
0·00625	2·263 „	3·80 „	0·029	0·88	0·42	0·33	0·691	1·08 „	0·80	1·01
0·00312	3·806 „	2·26 „	0·014	0·90	0·51	0·41	0·805	1·16 „	0·74	0·92

Calculated from the hydrolysis of K_3PO_4 at 20°.

0·0250	$0·930 \times 10^{-12}$	$9·25 \times 10^{-3}$	0·124	0·80	0·24	0·19	0·468	$1·04 \times 10^{-2}$	0·83	1·06
0·0125	1·431 „	6·01 „	0·065	0·84	0·33	0·26	0·572	1·02 „	0·84	1·07
0·00625	2·278 „	3·77 „	0·029	0·88	0·43	0·33	0·685	1·04 „	0·82	1·04
0·00312	3·858 „	2·23 „	0·015	0·91	0·51	0·41	0·784	1·00 „	0·85	1·04

Calculated from the hydrolysis of Na_3PO_4 at 38°.

0·0250	$2·591 \times 10^{-12}$	$1·29 \times 10^{-2}$	0·115	0·80	0·24	0·19	0·648	$3·02 \times 10^{-2}$	1·11	1·40
0·0125	4·203 „	0·797 „	0·056	0·84	0·33	0·27	0·759	3·07 „	1·09	1·33
0·00625	7·202 „	0·465 „	0·027	0·88	0·43	0·34	0·846	3·23 „	1·03	1·31
0·00312	1·305 „	0·257 „	0·013	0·91	0·52	0·43	0·914	3·33 „	1·00	1·22

Calculated from the hydrolysis of K_3PO_4 at 38°.

0·0250	$2·681 \times 10^{-12}$	$1·26 \times 10^{-2}$	0·115	0·80	0·24	0·19	0·629	$2·70 \times 10^{-2}$	1·24	1·57
0·0125	4·312 „	0·77 „	0·056	0·84	0·33	0·27	0·740	2·71 „	1·24	1·52
0·00625	7·261 „	0·46 „	0·027	0·88	0·43	0·34	0·839	3·01 „	1·12	1·41
0·00312	1·332 „	0·25 „	0·013	0·91	0·52	0·43	0·887	2·39 „	1·39	1·68

Table II. *The tertiary dissociation constant of phosphoric acid.*

Calculated from K_2HPO_4—K_3PO_4 mixtures

$$K_3 = \frac{[H^+]\left[B - \frac{[OH^-]}{\gamma_B} + \frac{[H^+]}{\gamma_S}\right]}{C - B + \frac{[OH^-]}{\gamma_B} - [H^+]}.$$

C	B	p_H	H^+	OH^-	μ	γ_B	γ_S	γ_T	$K_a' \cdot 10^{-12}$
0·0313	0·0062	11·20	$6{\cdot}31 \times 100^{-2}$	0·0016	0·11	0·80	0·25	0·20	1·06
0·0294	0·0118	11·55	2·82 „	0·0036	0·15	0·76	0·21	0·16	1·10
0·0278	0·0168	11·80	1·59 „	0·0063	0·12	0·78	0·23	0·19	0·94
0·0263	0·0211	11·95	1·12 „	0·0089	0·12	0·79	0·23	0·19	0·84

μ is the total ionic strength. The ionic strength is equal to the stoichiometric molarity of each ion multiplied by the square of its valence. The total ionic strength is equal to the sum of the strengths of the anions and cations divided by two. The activity coefficient of a strong electrolyte is the same in all dilute solutions of the same ionic strength.

K_h is calculated from equation (4), K_a from equation (5), and K_a' from equations (6), (7) or (8). K_w at 20° is $0{\cdot}86 \times 10^{-14}$ and at 38° is $3{\cdot}35 \times 10^{-14}$.

Discussion of K_3 calculated.

The tertiary dissociation constant may be calculated from the data obtained by the electrometric titration of phosphoric acid and from the formulae developed by Van Slyke [1922]. The equation for the dissociation of a weak acid in an alkaline system is No. 29 of Van Slyke's paper,

$$K_a' = \frac{[H^+]\left(B - \frac{[OH^-]}{\gamma_B} + \frac{[H^+]}{\gamma_S}\right)}{C - B + \frac{[OH^-]}{\gamma_B} - [H^+]}.$$

Table III of that paper gives K_a' calculated from the titration data of W. M. Clark for $H(K_2PO_4) + KOH$. The above equation was used in its simplified form assuming complete dissociation. A recalculation of K_2' using activity coefficients at the respective ionic strengths is given in Table III. These values are concordant with those obtained from the hydrolysis data.

The apparent tertiary constant may also be calculated from the apparent secondary dissociation constant knowing the hydrion concentration which defines the secondary phosphate. Replacing the secondary and tertiary salt concentrations in equation (6) by their equivalents,

$$[Na_3PO_4] = \frac{K_3'}{K_3' + [H^+]} \cdot [C],$$

$$[Na_2HPO_4] = \frac{K_2'}{K_2' + [H^+]} \cdot [C]$$

and solving the quadratic, we obtain,

$$K_3' = \frac{[H^+]^2}{K_2'}.$$

Therefore, the secondary phosphate ratio has a maximum when $[H^+]$ is the geometric mean of the apparent dissociation constants,

$$p_H = \frac{p_{K_2'} + p_{K_3'}}{2}.$$

The p_H of the secondary phosphate is difficult to localise experimentally. Clark's titration curve gives p_H 9·3 for 0·066 *M* K_2HPO_4, $p_{K_2} = 6·8$ and hence p_{K_3} is 11·8. The secondary phosphates prepared in this work had a p_H of 9·35 and hence p_{K_3} is 11·9.

Table III. *The tertiary dissociation constant of phosphoric acid.*

Calculated from titration data of W. M. Clark for $K_2HPO_4 + KOH$.

C	B	p_H	H	OH⁻	U	γ_B	γ_S	γ_T	$K_a' . 10^{-12}$
0·0313	0·0062	11·20	$6·31 \times 100^{-2}$	0·0016	0·11	0·80	0·25	0·20	1·06
0·0294	0·0118	11·55	2·82 „	0·0036	0·15	0·76	0·21	0·16	1·10
0·0278	0·0168	11·80	1·59 „	0·0063	0·12	0·78	0·23	0·19	0·94
0·0263	0·0211	11·95	1·12 „	0·0089	0·12	0·79	0·23	0·19	0·84

Conclusion.

The tertiary dissociation constant of phosphoric acid, determined from the hydrolysis of the tertiary phosphates, has been found to be $1·02 \times 10^{-12}$ at 20° and $1·48 \times 10^{-12}$ at 38°.

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LXVIII. THE CATALYTIC ACTION OF CUPRIC SALTS IN PROMOTING THE OXIDATION OF FATTY ACIDS BY HYDROGEN PEROXIDE.

By MARION ALICE BATTIE and IDA SMEDLEY-MACLEAN.

From the Lister Institute, London.

(*Received June 4th, 1929.*)

During the last few years experiments on the oxidation of the higher fatty acids by means of hydrogen peroxide in the presence of ferrous iron have been carried out in this laboratory by C. G. Daubney; the results however were found to be exceedingly irregular. Under conditions which were apparently similar, in some experiments oleic acid was rapidly attacked and largely broken down to carbonic, formic, acetic and succinic acids; in other experiments hardly any action took place. The alteration in the conditions, however, which so greatly increased the oxidation eluded discovery. In a further attempt to solve this problem, the present authors endeavoured to investigate the effect of the addition of small quantities of impurities to the reaction mixture.

The conditions of the original experiments were as follows. Into a large flask were introduced 2·5 g. oleic acid, 10·6 cc. *N* sodium or ammonium hydroxide and 125 cc. distilled water: when the oleic acid was dissolved, 15 cc. of a solution containing 0·613 g. ammonium ferrous sulphate and 350 cc. hydrogen peroxide solution (20 vols.) were added to the soap solution. The flask fitted with a reflux condenser was left for 2 hours in a bath at 60°, the outlet being connected to an apparatus for the absorption of CO_2. At the end of this time, 2 cc. concentrated sulphuric acid were added and nitrogen was blown through the apparatus for a further 2 hours. Any insoluble acid was then filtered off and the liquid steam-distilled.

We first investigated the effect of the addition of small amounts respectively of nickel, manganese, zinc and copper salts which were added to the mixture at the same time as the ferrous salts. It was at once evident that the reaction was influenced by the addition of the copper salt but no certain effect was noticeable when nickel, zinc or manganese salt was added. Next we tried the effect of replacing the ferrous salt by an equivalent amount of cupric salt and we found that consistent results were obtained. The oxidation was very much more marked than in the "inactive" experiments with the ferrous salt, but the proportion of volatile acid was never quite as great as in the "active" iron experiments. Since we had now found conditions which were always repeatable, and since under the influence of the cupric salt at 60° in the presence of excess of hydrogen peroxide oleic acid may be largely

broken down into carbonic, formic, acetic and succinic acids, we decided to investigate this reaction quantitatively. We do not, however, think that the condition in the original experiments with ferrous salts which determined their activity was the presence of copper salts.

The influence of copper salts in promoting oxidation.

A study of the literature showed that the action of cupric salts in promoting the oxidation of tincture of guaiacum in the presence of hydrogen peroxide had been frequently noted and that one or two other instances of their action had been observed. Bourquelot and Bougault [1897] first recorded that in the presence of copper salts tincture of guaiacum is coloured blue, just as it is by the action of oxidising ferments. Here the oxygen necessary for the oxidation is derived from the air, and the copper salt behaves as an oxidase. Thus, if to a 2 % solution of quinone two or three drops of a 1 % solution of copper acetate are added, the characteristic blue colour is produced slowly in the cold, more rapidly on heating. The presence of oxygen is essential and the colour is not produced *in vacuo*. Labat [1908] recorded that an intense red colour was produced with the Kastle-Meyer reagent (an alkaline solution of phenolphthalein decolorised by boiling with zinc dust) in the presence of hydrogen peroxide to which a drop of a 2 % solution of copper sulphate in glycerol had been added. Mueller [1918] again confirmed the observations of Bourquelot and Bougault and drew attention to the behaviour of cupric salts both as oxidases, the supply of oxygen being obtained from the air, and as peroxidases, hydrogen peroxide acting as the source of the oxygen used in the reaction. Mueller suggested that the cupric salt reacts with the hydrogen peroxide, one oxygen atom of the peroxide entering into combination with the partial valencies of the copper and that this unstable compound then reacts with the oxidisable compound.

Thomas and Carpentier [1921] repeated Labat's observation and showed that the Kastle-Meyer reagent is extraordinarily sensitive to the action of copper salts, the colour being perceptible with a solution of copper containing only one part in a hundred million; indeed the distilled water found in most laboratories where copper stills are used for its preparation was found to be active. The close resemblance of this action to that of a peroxidase was again stressed by these authors, who also made the interesting observation that the lactate and tartrate of copper were inactive, but that the acetate and butyrate were active, behaving like the mineral salts. Aloy and Valdiguié [1923] extended these observations and brought forward evidence in support of the view that the intermediate substance concerned in this reaction is an unstable cupric peroxide: this substance they prepared by the method originally described by Thénard and also by the addition of a solution of hydrogen peroxide to one of ammoniacal copper sulphate, and they found that the peroxide behaved as a peroxidase. The activation of oxygen by metallic copper was studied by Wieland [1923]; in his experiments various organic

acids, acetic, pyruvic, succinic, malic, benzoic, oxalic and lactic, were oxidised with the production of carbonic acid, the acids being shaken with oxygen in the presence of metallic copper. He found that the reaction only took place in acid solution and that when the metal had dissolved in the acid oxidation was at an end. He found a similar effect when cuprous salts were added in the presence of hydrogen peroxide and here again the effect was only produced in acid solution and came to an end when the cuprous salt was converted to the cupric state. He says "Vor allem aber büsst das peroxydische oder höherwertige Kupfer nach Abgabe eines Sauerstoffaequivalentes als Cuprisalz sein Oxydationsvermögen ein."

With regard to this we can only say that under the conditions of our experiments the cupric salts are certainly the active agents. Since, as far as our investigations go, the presence of an acid group in the molecule seems essential in order that oxidation may take place, it seems to us possible that the copper enters into the molecule of the oxidisable acid, forming a salt, and that the partial valencies of the copper then unite with the oxygen of the hydrogen peroxide as Mueller suggested, forming an unstable intermediate compound.

The close analogy of the action of cupric salts with that of the peroxidases made us hopeful that the oxidation of the higher fatty acids by means of hydrogen peroxide in the presence of cupric salts might present a closer analogy with the physiological process than oxidation by any other known laboratory method. When the higher fatty acids are acted upon by hydrogen peroxide in the absence of a catalyst they are very little attacked and therefore it has been difficult to establish the nature of the reaction. We decided therefore first to work out the method on the simpler fatty acids and their oxygenated derivatives and later to apply the method to a comparison of the oxidation of palmitic, oleic and stearic acids.

Preliminary experiments on the oxidation of oleic acid indicated that among the products of the reaction were acetic and succinic acids. The behaviour of these acids under the same conditions of oxidation was therefore examined and the reaction extended to other acids which might conceivably occur as intermediate products. Succinic acid was chosen for the investigation of the conditions of the reaction.

Influence of the reaction of the medium. In alkaline or neutral solution very little oxidation of succinic acid occurs; favourable results were obtained when the acidity of the mixture corresponded to 0·010 to 0·020 *N*. It seems essential that the reaction shall be acid but small changes in the hydrogen ion concentration appear to exert little influence.

Method.

2·5 g. of the acid to be oxidised were placed in a large flask and the calculated quantity of *N* NaOH solution was added, in most cases sufficient to make the whole reaction mixture equivalent to an acidity of 0·015 to

0·020 *N*. A measured quantity of a 0·73 % solution of crystalline cupric sulphate and 350 cc. of a previously neutralised solution of hydrogen peroxide (20 vols.) were then added, the latter reagent being present therefore in large excess. The mixture was left in a bath at 60° for a stated time, the outlet of the flask being connected with an absorption apparatus containing a known amount of a standard baryta solution: 2 cc. concentrated sulphuric acid were added to the contents of the flask and nitrogen was blown through for 1½ hours, the bath being meanwhile allowed to cool. When the oxidisable acid is insoluble in water, the addition of the sulphuric acid precipitates any insoluble acid and so prevents the further action of the peroxide. In the case of soluble acids, however, the effect of the hydrogen peroxide on the acid continues though at a lower temperature and in a much more acid medium during the time in which the carbonic acid is being blown off.

Table I.

(*a*) during time of experiment;
(*b*) during subsequent steam-distillation at 100°.

Succinic acid g.	Time of exp. hr.	Reaction of mixture	Atomic proportion Cu to 1 mol. succinic acid	Percentage of original succinic acid appearing as CO_2 (*a*)	CO_2 (*b*)	Formic	Acetic
Influence of reaction of medium:							
		Alkali					
2·5	2	0·054 *N*	0·000	4·0	—	—	—
,,	1	,,	0·077	0·6	—	—	—
,,	0·5	,,	0·077	0·8	—	—	—
		Acid					
,,	1	0·015 *N*	0·000	7·1	37·1	—	—
,,	1	,,	0·077	45·2	6·0	—	—
,,	0·5	,,	0·077	41·2	8·0	3·9	26·5
Influence of proportion of copper:							
		Acid					
2·5	0·5	0·012 *N*	0·000	Trace	—	—	—
,,	,,	0·015 *N*	0·026	9·4	—	—	—
,,	,,	0·018 *N*	0·052	28·8	15·0	5·0	22·0*
,,	,,	0·020 *N*	0·078	53·4	5·0	2·0	17·6*
Influence of time of experiment:							
		Acid					
2·5	0·5	0·014 *N*	0·026	9·7	—	—	—
,,	1	,,	,,	29·6	—	—	—
,,	1·5	,,	,,	48·9	—	—	—
,,	2	,,	,,	56·9	—	—	—

* In these experiments the hydrogen peroxide was removed before the steam-distillation.

The acid mixture is then filtered. In the earlier experiments the solution was then steam-distilled, but in this case the excess of hydrogen peroxide continues to act at a higher temperature on any products in the solution. In the later experiments, therefore, before carrying out the steam-distillation, the excess of hydrogen peroxide was destroyed by making the solution alkaline, adding to it finely powdered charcoal and allowing it to stand overnight.

The next morning the filtered solution was again acidified and steam-distilled, the distillate collected, any emergent gas being passed through a standard baryta solution. The distillate was neutralised with a known amount of *N* NaOH solution and evaporated to dryness. The amount of formate in the sodium salts of the volatile acids was estimated, and the molecular weight determined. In most cases the acid is completely soluble and appears to consist almost entirely of acetic and formic acids. Aldehyde was usually present and was estimated in an aliquot part of the original distillate. The results of the experiments with succinic acid which are collected in Table I show (1) that the addition of the cupric salt is effective in acid but not in alkaline solution; (2) that after the mixture has stood for half an hour at 60°, about 75 % of the succinic acid has been decomposed into carbon dioxide, formic acid and a notable proportion of acetic acid.

Comparison of the relative effect of ferrous, cupric and cuprous salts.

Neither in acid nor in alkaline solution did we find that ferrous salts influenced the oxidation of succinic acid under the conditions of our experiments. The amount of oxidation was here measured by the percentage of the original acid appearing as CO_2 during the time of the experiment. It will be seen that the results of the experiments in which iron was present were similar to those in which no metallic salt was added. The possibility that intermediate products of oxidation were present was not however excluded.

Table II. *Showing the comparative effect of cupric and ferrous salts on the oxidation of succinic acid by hydrogen peroxide.*

Succinic acid g.	Time of exp. hr.	Reaction of mixture (acid)	Metal added	Percentage of original succinic acid converted to CO_2
2·5	1	0·022 *N*	—	7·2
,,	0·5	0·014 *N*	Fe	6·0
,,	0·5	,,	Fe	8·8
,,	1	,,	Fe	5·2
,,	1	,,	Fe	4·4
,,	0·5	,,	Cu	41·2
,,	1	,,	Cu	50·4
,,	1	,,	Cu	46·4
,,	1	,,	Cu	54·0
,,	1	,,	Cu	45·2

0·078 of an atomic proportion of $Cu^{''}$ or $Fe^{''}$ was taken in each case.

The substitution of cupric chloride or cuprous chloride for cupric sulphate gave similar results. The action of the cuprous chloride was rather slower than that of the cupric compound. The effect of the same salts in promoting the decomposition of the hydrogen peroxide was then studied, the decomposition being measured by the volume of oxygen evolved in the same time under similar conditions at 60°.

70 cc. hydrogen peroxide were neutralised with 1·4 cc. *N* NaOH, 23·6 cc. water and 2 cc. *N* acid added and equivalent amounts of cupric and

cuprous chloride and of ferrous sulphate respectively. The relative amounts of oxygen liberated in the same period of time when measured under similar conditions were respectively 910 cc. ($CuCl_2$), 610 cc. (CuCl) and 215 cc. ($FeSO_4$). The control in the absence of metallic salts liberated 160 cc.

It appears therefore that cuprous salts are more effective in bringing about the decomposition of hydrogen peroxide but that cupric salts are more active in catalysing the oxidation of the aliphatic acids by hydrogen peroxide.

We then investigated the effect of the same oxidising agent on some of the acids which might possibly occur as oxidation products of succinic acid and the results which we obtained are set forth in the following table (Table III). In each case the reaction of the mixture was 0·014 *N* acid and 0·078 of an atomic proportion of copper was present for each molecular proportion of the acid to be oxidised: the conditions of the experiments were similar to those already described. The action was estimated by the weight of carbonic acid evolved during the time of the experiment and during the subsequent period in which the carbonic acid is being blown off from the solution. It is possible that, in the absence of the cupric salts, oxidation products containing the same number of carbon atoms may have been produced. We did not however obtain any evidence of this.

Table III.

			H_2O_2 and Cu		H_2O_2 alone	
Acid	g.	Time of exp. hr.	Weight CO_2 g.	Acid converted to CO_2 %	Weight CO_2 g.	Acid converted to CO_2 %
Succinic	2·5	0·5	1·54	41·2	—	Trace
		1	1·68	45·2	—	7·1
			2·01	54·0	—	—
			1·58	42·4	0·04	1·5
			1·87	50·2	—	—
			1·65	44·4	0·006	0·02
Malic	1·0	1	0·77	58·8	0·03	2·3
Tartaric	2·5	0·5	>1·68	>57·3	CO_2 evolved too rapidly for complete absorption	
		0·75	>1·93	>65·8		
	1·0	0·5	1·18	100	0·0	0·0
		1	1·18	100	0·0	0·0
Dihydroxymaleic	1·0	1	0·76	64·0	0·29	24·45
	„	1·25	0·72	60·2	0·34	28·60
Pyruvic	1·0	1	0·90	60·00	0·31	21·00
	„	—	0·85	56·67	—	—
Lactic	1·0	1	0·63	43·3	0·002	0·14
	„	—	0·56	38·0	—	—
Malonic	1·0	1	0·02	1·8	—	—
	„	1·25	0·48	38·5	—	—
	„	2	0·43	34·6	0·01	0·8
Acetic	2·5	1	1·22	33·3	—	—
	1·0	1	0·47	32·3	0·007	0·4

When tartaric acid is oxidised by means of hydrogen peroxide in the presence of ferrous iron, dihydroxymaleic acid is the first product of oxidation [Fenton, 1894, 1896]. Since, however, in the presence of cupric salts, dihydroxy-

maleic acid is much less readily oxidised to carbon dioxide than is tartaric acid, the evidence available does not support the view that, in the presence of cupric salts, tartaric acid is first converted to dihydroxymaleic acid. Malonic acid showed a curious period of lag before action began in three of our experiments, during the first hour there was practically no evolution of carbonic acid, then in the next 15 minutes a rapid evolution of gas took place. The notable proportion of acetic acid formed during the oxidation of succinic acid was interesting, and the fact that acetic acid itself was also converted to carbonic acid, one-third of the amount taken being completely oxidised to carbonic acid in an hour, was also noteworthy.

In the absence of the cupric salt only two of the acids taken were oxidised by the action of the hydrogen peroxide under the conditions of our experiments. These were pyruvic and dihydroxymaleic acids, but the evolution of carbon dioxide is much greater in the presence of the copper salt.

Similar experiments were carried out using alcohol, acetone and glycol as the materials to be oxidised, but in no case was there any evidence of oxidising action taking place. The addition of dilute sulphuric acid to the mixture produced no effect, and the action of the cupric salt seems only effective when the substance to be oxidised is an acid and the experiment is carried out when a certain proportion of the free organic acid is present.

The oxidation of the higher fatty acids is at present under investigation, but as the method seems to be of general application for the oxidation of the aliphatic acids, we desire to record these preliminary results.

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LXIX. AN IMMEDIATE ACID CHANGE IN SHED BLOOD.

By ROBERT EMLYN HAVARD[1]
and PHYLLIS TOOKEY KERRIDGE[2].

From the Medical Unit Laboratory, London Hospital, and the Department of Physiology and Biochemistry, University College, London.

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Introduction.

The p_H of blood was measured at body temperature by the glass electrode method about 2 minutes after the withdrawal of the blood from the blood-vessel. The p_H was observed to decrease by about 0·05 a few minutes later. This acid change seemed to be very important, as it meant that later determinations do not represent the p_H of the blood in the body. A typical p_H-time curve is shown in Fig. 1, curve 1. This p_H change was investigated further.

Technique.

(*a*) *Collection of blood.*

Human blood was mainly used in this research, the total number of individuals from whom one or more blood samples were taken being 21. No special attempt was made to control the physiological conditions which might affect the c_H of the blood, but the subject sat still for 5–10 minutes before the blood-vessel was punctured. Stasis was generally avoided, but in the later experiments it was used when convenient.

The skin was cleaned with an antiseptic, the vessel punctured with a sterile needle, and the blood was drawn into a syringe. Contact of the blood with air was avoided by filling the dead space of the syringe and needle with liquid paraffin. Change of temperature during this manipulation was reduced to a minimum. This could be done by using a small all-glass syringe, previously warmed to 38°. When a syringe with metal plunger and cap was used it was necessary to warm it to about 45°, and to lag it with cloth, as otherwise the conductivity of the metal caused it to cool quickly.

In the experiments in which chemical substances in solution were added to the blood, these were measured into the syringe before the puncture. The

[1] Beaney Research Student, Guy's Hospital.
[2] Working for the Medical Research Council.

presence of a glass bead proved essential to ensure complete mixing. No liquid paraffin was used in these cases (except as a piston lubricant). The needle was inserted into the vessel, and blood allowed to flow through it before the syringe was attached. In this way air was removed from the needle and there was no danger of traces of the chemical entering into the circulation.

(b) Measurement of p_H.

The hydrogen ion concentration of the blood was measured by the glass electrode method [Kerridge, 1926]. The blood was transferred immediately from the warm syringe to a standardised electrode, which was at body temperature, and the first c_H determination completed between one or two minutes after the blood left the vessel.

Two completely independent glass electrode apparatus were used, so that simultaneous p_H-time curves could be obtained from samples of blood either at two different temperatures, or with and without added substances. The calomel and glass electrodes of each apparatus were enclosed in a large air-bath with automatic temperature regulation and an efficient fan. The air-baths were lined with metal, which was connected to earth. When the door of an air-bath was opened, as for the introduction of the sample of blood, the fan was stopped, and an accessory heating lamp, situated near the door, was turned on. It was very important that this procedure should be completed as quickly as possible, and the fan turned on again as soon as the door was shut. The temperature remained constant within 0·5° under these circumstances. The glass electrodes were all made of Wood's glass of the formula recommended by Hughes [1928]. They were standardised daily against a solution of $M/20$ potassium hydrogen phthalate, the p_H of which was taken to be 3·97°, both at room temperature and at 37·5°. The standardisations were carried out at the same temperature as the subsequent experiment. From time to time, the electrodes were checked by determining the p_H of a dilute phosphate buffer solution, of about p_H 7·3, which was also estimated by the quinhydrone electrode method. The calomel electrodes contained 3·5 M KCl, and the potassium phosphate buffer used on one side of the glass membrane was dilute (approx. $M/20$). Under these circumstances the liquid-liquid junction potentials can be neglected, but evidence was obtained which showed that this is by no means the case when the calomel electrodes contain $M/10$ KCl and the phosphate buffer solution is concentrated. Special care was taken with the ground caps of the calomel electrodes, as KCl may diffuse into the electrode during long experiments when these fit badly.

The Lindemann electrometers were adjusted so that a deflection caused by 0·25 mv. (corresponding to 0·004 p_H) could be detected. By the kind permission and courteous cooperation of the Cambridge Instrument Co., both potentiometers were calibrated at their works for zero error and uniformity of slide wire against one of their standard potentiometers. The potentiometers

were standardised before each experiment with a Weston cadmium cell. The error in the potentiometer readings is estimated at 0·5 %. Control experiments in which the p_H of the same liquid was measured in the two apparatus agreed within 0·02 p_H. This difference gives the order of the combined instrumental and manipulative errors. The control experiments were done both on buffer solutions, whose p_H was checked by the quinhydrone electrode method, and on blood. Thus it may be said that the absolute p_H measurements were accurate to 0·02 p_H, but that differences of 0·005 p_H on any one sample were significant.

Experiments.

(i) *Effect of sodium fluoride.*

Evans [1922] has described changes in blood shortly after shedding, which were stopped by sodium fluoride. We found that addition of 0·02 % sodium fluoride to the blood caused an almost complete disappearance of the change in p_H corresponding to the part of the p_H-time curve denoted at *DE* in Fig. 1. The inhibition was complete in greater concentrations, and we used 0·06 % in subsequent experiments in which we wished to eliminate this p_H change (cf. Fig. 1, curve 2).

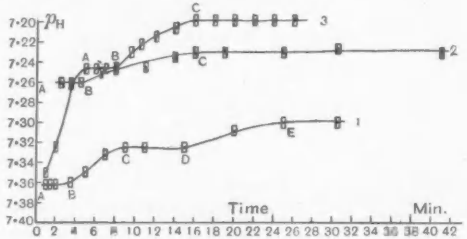

Fig. 1. p_H-time curves of shed blood.

Curve 1. Venous blood (human).

Curve 2. Venous blood (human) + 0·06 % sodium fluoride.

Curve 3. Arterial blood (cat under chloroform) + 0·04 % potassium oxalate + 0·06 % sodium fluoride: kept at 0° until 1 min. before experiment.

The abscissae represent time in minutes after the blood left the vessel in curves 1 and 2, and after the blood was put in the electrode in curve 3. The rectangles represent size of experimental errors.

AB corresponds to "latent period."

BC corresponds to "first acid change."

DE corresponds to acid change inhibited by sodium fluoride.

We propose to use in the remainder of this paper the term "first acid change" for the change in p_H corresponding to the part of the curve in Fig. 1 labelled *BC*. It is this change which has occupied most of our attention. The time before the beginning of the first acid change, labelled *AB*, will be referred to as the "latent period."

(ii) *The "first acid change."*

Particulars of p_H-time curves at 38° and 28° are given in Table I. Both the latent period and the duration were greater at the lower temperature, although they varied between rather wide limits. No figure has been given for the latent period of venous blood at 38° as in 12 out of 31 experiments the "first acid change" had apparently commenced before the first p_H reading was taken about 2 minutes after shedding; of the remainder, 10 latent periods were between 2 and 8 minutes, and the others had widely different values up to a maximum of 40 minutes.

A decrease in p_H of 0·05 corresponds to a 12·5 % increase in hydrogen ion concentration, and an average deviation from the mean of 0·015 p_H, to a 3·5 % variation. The variation is of the same order when only repeated determinations on the blood of a single individual are considered.

There were no significant differences between the results on arterial and on venous blood.

Table I.

	Venous blood		Venous blood +0·04 % K oxal. +0·06 % NaF		Arterial blood
Temperature	38°	28°	38°	28°	38°
Latent period (min.):					
Mean	—	11	6	19	7·5
Maximum	40	13	14	30	17
Minimum	1·5	8	3	12	2·5
First acid change (p_H):					
Mean	0·055	0·05	0·04	0·05	0·05
Maximum	0·095	0·06	0·08	0·09	0·08
Minimum	0·02	0·03	0·015	0·03	0·025
Average deviation from mean	0·015	—	0·01	0·02	0·015
No. of experiments	31	3	9	7	7
Duration of first acid change (min.):					
Mean	9	5	10	18	5
Maximum	21	9	15	30	9
Minimum	2	2·5	4	2	2·5

At room temperature (19·5° ± 1·5°) there was no significant change in p_H, greater than the experimental error, within about 1½ hours after the blood was shed, but then an acid change took place very slowly, after which the p_H again became constant.

(iii) *Effect of anticoagulants.*

In the course of the experiments already described the blood clotted in the electrode. The following anticoagulants were added to the blood, without affecting the "first acid change":

Heparin 0·02 %, 0·04 % and 0·08 %; sodium fluoride 0·3 %; ammonium oxalate 0·045 %; potassium oxalate 0·04 %.

The substances, dissolved in isotonic saline, were added to the blood in such concentration as to give a 10 % dilution of the blood. Control experiments,

in which dilution was made with isotonic saline alone, showed no measurable change in the initial p_H of the blood or in the "first acid change."

Heparin (0·02, 0·04 or 0·08 %) and sodium fluoride (0·06 %) added to the blood together do not inhibit the acid change corresponding to the part of the curve in Fig. 1 labelled *DE*, which is inhibited by sodium fluoride alone. We have no explanation to offer.

Data referring to the "first acid change" in the presence of potassium oxalate (0·04 %) and sodium fluoride (0·06 %) are given in Table I, columns 3 and 4. It will be noted that the mean latent period is greater than in the case of blood alone, and that the mean size of the "first acid change" is less, although the difference in dimension is of questionable significance.

(iv) *Effect of potassium cyanide and of thymol.*

Potassium cyanide was added in $M/100$ concentration, without affecting the shape of the p_H-time curve. The cyanide made the initial p_H more alkaline, unless it was previously neutralised. Thymol ($M/500$) likewise had no effect.

(v) p_H*-time curves of plasma.*

The latent period of the "first acid change" of venous blood containing potassium oxalate (0·04 %) and sodium fluoride (0·06 %) had been found to have a mean value of 19 minutes at 28°. In another series of experiments the blood was centrifuged at 28° during this latent period, and the plasma was separated and placed in the electrode. In a typical experiment centrifuging was carried out between the fourth and eighth minutes, and the first p_H measurements were made at the tenth minute after the blood was shed. The plasma p_H-time curves showed no "first acid change." Corpuscles with adherent plasma showed typical p_H-time curves with a "first acid change." Plasma to which corpuscles were added to give one-quarter of the normal number showed curves with a typical "first acid change."

(vi) *Effect of laking the blood.*

p_H-time curves on laked blood were made from venous blood containing potassium oxalate (0·04 %), sodium fluoride (0·06 %) and saponin (1 %). These showed typical "first acid changes." The same result was obtained when laked blood was centrifuged at room temperature, before the experiment, to free the fluid from white cells.

(vii) *Miscellaneous experiments.*

Any possible unexplained electrical effects of the fibrin or of the corpuscles settling on to the glass membrane were ruled out as the cause of the "first acid change" by reversing the customary arrangement of buffer and blood in the electrode. The "first acid change" still occurred when the corpuscles dropped away from the membrane.

Two samples of blood with potassium oxalate and sodium fluoride kept at 38° for 0·5 hour, and for 1 hour respectively, before transference to the

glass electrode, showed no change of p_H with time. Their p_H agreed with the final p_H of another sample of blood which had been placed in the electrode as soon as possible after it was shed, and which had undergone a typical "first acid change" in the electrode. Hence in the first two cases the "first acid change" may be presumed to have taken place while the blood was still in the syringe, and to be quite independent of the electrode. Similarly blood containing heparin and hydroxyquinoline potassium sulphate (as an antiseptic) showed no change of p_H with time after 22·5 hours at 38°.

Estimations were made of the total carbon dioxide and of the oxygen contents of blood before and after the "first acid change". In these experiments a sample of blood was drawn into a syringe at 0°, which contained potassium oxalate and sodium fluoride. One portion was taken immediately for analysis. The remainder was warmed to 38° and kept at that temperature for 0·5 hour before an analysis was made. The method of analysis used was that of Harington and Van Slyke [1924]. No appreciable difference between the O_2 and total CO_2 contents of the two samples could be detected.

Samples of blood were taken in a similar way for lactic acid estimations. These were kindly made for us by Dr H. D. Kay by the method of Friedemann, Cotonio and Shaffer [1927], on the ether extract of a trichloroacetic acid filtrate of the blood. No significant difference between the results on the two samples was observed. However, it is possible that the "first acid change" might be occasioned in the first sample in the course of the precipitation with trichloroacetic acid, and it is therefore impossible to pronounce definitely that the acid which causes the shift in hydrogen ion concentration is not lactic acid.

Discussion.

The results of the experiments described in the previous section may be summarised as follows. The "first acid change" is not associated with the clotting of blood; it is delayed by decrease of temperature; it is not affected by substances which inhibit oxidation phenomena (KCN), enzyme action (thymol), or glycolysis (NaF). It does not occur in plasma, but does occur when the red corpuscle wall is broken down and in the absence of leucocytes. No explanation has been found.

There are references in the literature to changes in p_H accompanying clotting. Kugelmass [1922] reported an alkaline change of 0·3 p_H, using a hydrogen electrode on a solution of diluted oxalated plasma. Hirsch [1924] found an acid change of 0·09 p_H in rabbit's plasma by a colorimetric method. Ross [1921] found no change in the p_H of blood on clotting. Our experiments confirm those of Ross, as the p_H-time curves of blood at 38° and 28° were unaffected by the addition of anticoagulants, and as in the experiments at room temperature the blood clotted in the electrode, while the p_H remained constant.

The existence of this unexplained "first acid change" signifies that p_H measurements of blood at 38° made more than about 4 minutes after the

blood is shed will be more acid than when it leaves the blood-vessel, by an average of 0·05 p_H. This appears to be due to an increase in acidity within the red corpuscle, as it occurs in centrifuged laked blood, but not in plasma. From the buffer curves of blood of Van Slyke, Hastings, and Neill [1922], it can be calculated that an increase in acidity of 0·05 p_H corresponds to an addition of 1·2 millimol of acid per litre of blood. Assuming that this p_H change occurs at constant CO_2 content, it must be accompanied by a disturbance of the $\frac{[H_2CO_3]}{[BHCO_3]}$ ratio in the blood. The corresponding changes in $[H_2CO_3]$ and $[BHCO_3]$ can be calculated by the Henderson-Hasselbalch equation. At 38°, assuming pK = 6·17 and constant, the change in $[H_2CO_3]$ is 11 %, in $[BHCO_3]$ 5 %. Thus, blood having initial pCO_2 of 48 mm., and combined CO_2 of 45·5 vols. %, would have 53 mm. and 43·3 vols. % respectively after the "first acid change." Thus, delayed determinations of pCO_2 and vCO_2 of blood will misrepresent the amounts at the time of shedding by this amount. At 28°, as the CO_2 tension is lower, the difference in pCO_2 comes within the experimental error of the methods available, thus thwarting an attempt to demonstrate independently the "first acid change" by measuring the CO_2 tension of blood during the latent period, and after the acid change had taken place. A "first acid change" has been also observed in the blood of cats, dogs and rabbits. Experiments on the behaviour of blood in pathological conditions are in progress.

Precautions for measurement of p_H of blood.

There remains to be considered the best practical method of measuring the p_H of blood as it leaves the blood-vessel. It may be estimated at 38° within 1 or 2 minutes, with care taken to avoid cooling in transference from vessel to electrode. This, however, is not always convenient. Secondly, potassium oxalate (0·04 %) and sodium fluoride (0·06 %) may be added, the determination made less hurriedly and a correction of 0·05 p_H applied for the "first acid change." The average variation from the mean correction in normals is only $\pm$ 0·015 p_H, but nevertheless the method is objectionable as we have no evidence as to the size of the correction, or as to its variation in different physiological conditions, or in pathological states. A third method is to measure the p_H at room temperature, when no acid change occurs up to 1·5–2 hours after the blood is shed, and to apply a temperature correction. We have found the mean p_H of venous blood to be 7·32 at 38°, 7·47 at 28°, and 7·60 at 19·5°. These correspond to a change in p_H of 0·015 per degree. Martin and Lepper [1926] give 0·011 p_H per degree. A correction of this magnitude introduces a considerable degree of uncertainty, especially as nothing is known of its variation in different physiological and pathological conditions of the blood. A fourth method is as follows.

The blood is rapidly cooled by drawing into an ice-cold syringe, and is kept in the syringe on ice until convenient. (Care must be taken that the

temperature is not very much below 0°, or the blood will freeze, and lake on thawing.) The blood is brought to room temperature immediately before transference to an electrode at 38°, and a p_H-time curve taken. A typical form is shown in Fig. 1, curve 3. The first part shows a decrease of p_H with temperature which corresponds to the change in p_H incident to the warming of the blood. This is complete in about 4–5 minutes, and is followed by a steady p_H period. The "first acid change," which is apparently delayed by the cooling, then follows. The "plateau" lasts about 5 minutes, and has been shown to agree with the p_H of a sample of the same blood measured immediately after it was shed. In one experiment the blood was left for as long as 17 hours on ice before measurement. This last method is in our opinion the best, when the p_H cannot be measured at 38° immediately. It does not require the introduction of any correction, and can be done at any convenient time subsequent to the puncture. The addition of oxalate and fluoride is not essential.

Summary.

1. An increase in the hydrogen ion concentration of blood has been found to take place soon after the blood is shed.

2. This change has a mean value of $-$ 0·05 p_H at 38° and at 28°. It takes place within about 6 minutes at 38°, and 11 minutes at 28°. It does not occur until after 1·5–2 hours at room temperature.

3. The change is unaffected by sodium fluoride, anticoagulants, potassium cyanide or thymol. It takes place in laked blood and centrifuged laked blood but not in plasma.

4. It is suggested that blood should be cooled to 0° before subsequent p_H measurement at 38°. In this way the acid change can be delayed long enough to enable a satisfactory p_H estimation to be made.

We wish to thank Prof. J. Barcroft, Prof. Arthur Ellis, and Prof. C. Lovatt Evans for much helpful interest and criticism, Miss N. Henderson for assistance in the early part of the work, the Medical Research Council and the London Hospital Medical College Research Fund for defraying the expenses of the research, and the Cambridge Instrument Co. for the loan of instruments.

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LXX. STUDIES IN CARBOHYDRATE METABOLISM.

VI. THE ANTAGONISTIC ACTION OF PITUITRIN AND ADRENALINE UPON CARBOHYDRATE METABOLISM WITH SPECIAL REFERENCE TO THE GASEOUS EXCHANGE, THE INORGANIC BLOOD-PHOSPHATE AND THE BLOOD-SUGAR.

By CHARLES GEORGE LAMBIE and FRANCES AGNES REDHEAD.

From the Clinical Research Laboratory, Royal Infirmary, and the Research Laboratory of the Royal College of Physicians, Edinburgh.

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In recent years a considerable amount of attention has been directed to the question of the physiological antagonism between pituitrin and insulin, particularly in relationship to carbohydrate metabolism.

With the discovery of insulin opportunities were afforded for studying more directly the interaction of these hormones, and interest in the subject was stimulated afresh by the observations of Burn [1923], who demonstrated that pituitrin could inhibit the fall in blood-sugar following administration of insulin and bring about recovery from insulin hypoglycaemia in rabbits. These results were confirmed by numerous observers [Olmstead and Logan, 1923; Moehlig and Ainslee, 1925; Lawrence and Hewlett, 1925; Voegtlin, Thompson and Dunn, 1925; Geiling and Britton, 1927]. The suggestion put forward by Burn that these findings might be explained by a direct and specific antagonism between pituitrin and insulin has resulted in attempts being made by other workers to look for evidence of this antagonism in other directions. Thus Joachimoglu and Metz [1924] have employed the isolated uterus, but the evidence of antagonism was not conclusive. Serebrijski and Vollmer [1925] and Koref and Mautner [1926] found that when insulin was injected simultaneously with pituitrin it did not inhibit the antidiuretic effect of the latter, but during insulin hypoglycaemia the antidiuretic action was abolished. Given separately both insulin and pituitrin were antidiuretic, insulin causing water retention in the tissues, and pituitrin acting directly upon the kidneys. The peculiar and variable effect of insulin upon blood concentration, producing hydraemia or anhydraemia according to the conditions, is a factor which will have to be taken into consideration in

interpreting these results [Drabkin and Edwards, 1924; Levine and Kolars, 1924–1925; Villa, 1924; Olmstead and Logan, 1923; Haldane, Kay and Smith, 1924; Olmstead and Taylor, 1924].

Coope and Chamberlain have shown that pituitrin and insulin are antagonistic as regards certain changes in fat metabolism [Coope, 1925; Coope and Chamberlain, 1925].

The facts so far accumulated show that certain metabolic processes are influenced in opposite directions by insulin and pituitrin, and to this extent they may be said to be mutually antagonistic, but conclusive evidence of a direct inactivation of one hormone by the other has not been obtained. The changes which occur in fat metabolism and water metabolism under insulin may merely be secondary results of the action of insulin upon the combustion and storage of carbohydrate. With regard to carbohydrate metabolism, it may be noted that investigations regarding the modification of insulin action by pituitrin have been almost entirely confined to observations upon the blood-sugar or upon symptoms dependent upon changes in blood-sugar concentration, and the same question arises as to whether the fact that the blood-sugar concentration is altered in the opposite direction by insulin and pituitrin is to be explained by direct inactivation or by the result of some secondary effects of the hormone. In this connection it may be mentioned that Lambie [1926] and Clark [1928] have found that in the decerebrate cat, with the liver excluded from the circulation and constant transfusion of dextrose, pituitrin is unable to prevent the fall in blood-sugar caused by insulin, and this would suggest that, in the intact animal, either the liver is necessary to effect the mutual inactivation of insulin and pituitrin, or it is merely the source of dextrose yielded up under the influence of pituitrin to maintain the blood-sugar at or above the normal level in experiments in which the organ is intact. In so far, however, as changes in the concentration of the blood-sugar are not necessarily a measure of utilisation of sugar in the tissues, it cannot be inferred with certainty that even with the liver excluded from the circulation pituitrin is without influence upon carbohydrate metabolism.

In order, if possible, to throw light upon the effect of pituitrin on the metabolism of carbohydrate, it was thought desirable to study the respiratory metabolism and the inorganic blood-phosphate after administration of carbohydrate or insulin together with pituitrin, and the experiments described below were undertaken with that object.

If pituitrin inactivates insulin, changes of a similar character to those met with in diabetes might be looked for.

Hines, Leese and Boyd [1927], in experiments upon dogs with continuous transfusion of dextrose and pituitrin, came to the conclusion that while pituitrin caused less sugar to be retained than in controls, it was without effect upon the respiratory quotient and heat production: but the conditions of these experiments may not have been favourable for obtaining a positive result.

In view of the alleged capacity of pituitrin to annul the action of insulin upon carbohydrate metabolism, it was thought that an opportunity might also be afforded to study the metabolism of dihydroxyacetone under these conditions and thus gain further information upon the question as to whether this triose can be utilised even when the metabolism of dextrose is inhibited.

Since adrenaline is another hormone which, like pituitrin, has an action opposite to that of insulin, in so far as it can raise the blood-sugar and cause recovery from insulin hypoglycaemia, it was deemed of interest to carry out some experiments in order to determine how the changes in the respiratory metabolism compared with those obtained with pituitrin.

EXPERIMENTAL.

A. Respiratory exchange, blood sugar, and inorganic blood-phosphate in the human subject.

As in previous experiments [Lambie and Redhead, 1927] the subject was in the post-absorptive condition. The expired air was collected in 6-minute samples, using the Douglas bag method, and was analysed by means of the Haldane apparatus. The basal metabolic rate and fasting blood-sugar were first determined, after which the substance to be investigated was administered, and subsequently samples of expired air and blood were taken at intervals. The blood-sugar was estimated by the Hagedorn-Jensen method. When intravenous administration was employed, the sugar (20 g.) was dissolved in 100 cc. of saline and sterilised by filtering through a Seitz germicide (EK) filter No. 6 using pads impermeable to organisms. The injection was made at a uniform rate and occupied a period of exactly 7 minutes.

Exp. 1 (*a*). Respiratory metabolism and blood-sugar curve in normal student after intravenous injection of 20 g. of dextrose.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar g. per 100 cc.
0	+ 7	1·62	0·99	57·25	0·086
1–7	Dextrose intravenously				
1–3	+28	2·00	0·87	52·0	—
3–6	+ 6	1·72	0·73	45·0	—
10	+ 4	1·63	0·85	41·25	0·133
20	+11	1·73	0·89	44·0	0·108
30	+17	1·86	0·81	45·0	0·100
40	+ 8	1·72	0·82	44·75	0·087

Exp. 1 (*b*). Same as preceding + 0·75 cc. of pituitrin* administered subcutaneously while dextrose transfusion was proceeding. (Dextrose dissolved in 200 cc. saline.)

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar g. per 100 cc.
0	+ 3	1·56	0·88	76	0·079
1–7	Dextrose intravenously + pituitrin subcutaneously				
10	+17	1·81	0·79	71	0·166
25	+ 4	1·56	0·91	71	0·146
40	+ 7	1·62	0·90	70·71	0·139
50	+18	1·80	0·87	77·65	0·070
80	+15	1·72	0·95	77·5	0·097
120	+17	1·87	0·67	71·5	0·106

* The pituitrin used was that manufactured by Parke Davis and Co.

Exp. 2 (*a*). Respiratory metabolism in diabetic patient on low diet, after intravenous injection of 20 g. of dextrose.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.
0	− 9	1·27	0·72	36·66
1–7	Dextrose intravenously			
10	− 6	1·30	0·84	42·0
20	− 4	1·27	1·00	52·5
30	− 7	1·14	0·82	42·5
40	−24	1·09	0·72	37·0
50	−20	1·11	0·72	42·0

Exp. 2 (*b*). Same as preceding + 1 cc. pituitrin administered subcutaneously at end of dextrose injection.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.
0	+ 4	1·49	0·67	38·5
1–7	Dextrose intravenously + pituitrin subcutaneously			
10	+ 6	1·53	0·64	38·0
20	+ 7	1·56	0·65	38·5
30	+ 7	1·6	0·65	39·0
40	−16	1·18	0·75	37·0
50	−11	1·25	0·74	38·0

Exp. 3 (*a*). Respiratory metabolism in normal male after intravenous injection of 20 g. of dihydroxyacetone.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.
0	− 7	1·59	0·83	29·35
1–7	Dihydroxyacetone intravenously			
1–3	+15	1·94	0·87	42·0
3–6	+50	2·51	0·92	61·5
10	+49	2·54	0·86	57·0
20	+36	2·35	0·81	51·0
30	+12	1·91	0·84	47·0
40	+ 7	1·85	0·80	46·5
60	+ 3	1·80	0·76	40·25

Exp. 3 (*b*). Same as preceding + 1 cc. pituitrin.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.
0	+ 5	1·84	0·77	40·2
1–7	Dihydroxyacetone intravenously + pituitrin subcutaneously			
1–3	+10	1·91	0·78	40·5
3–6	+32	2·25	0·86	50·4
10	+17	1·97	0·89	52·25
20	+15	1·99	0·81	46·5
30	+35	2·40	0·71	48·0
40	+16	1·98	0·84	54·5
70	+19	2·01	0·89	52·0
100	+ 8	1·88	0·76	38·75

Exp. 4 (*a*). Respiratory metabolism, blood-sugar and inorganic blood-phosphate in normal male after injection of 15 units of insulin subcutaneously.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	−10	1·34	0·86	38·5	0·100	3·428
Insulin 15 units subcutaneously						
15	−18	1·20	0·92	37·0	0·095	3·387
30	− 2	1·45	0·87	43·0	0·085	3·170
45	−11	1·40	0·85	39·25	0·079	2·928
60	− 9	1·35	0·88	38·5	0·079	2·991

Exp. 4 (*b*). Respiratory metabolism in normal male after subcutaneous injection of 1 cc. of pituitrin.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	−20	1·20	0·72	36	0·099	3·377
Pituitrin subcutaneously						
10	−12	1·29	0·94	44	0·107	3·172
20	−10	1·34	0·85	42	0·112	2·906
40	−13	1·32	0·78	39·5	0·113	2·777
50	−15	1·31	0·79	37	0·107	2·777

Exp. 5 (*a*). Respiratory metabolism in normal male after subcutaneous injection of 10 units of insulin.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.
0	− 3	1·24	0·95	37·2
Insulin subcutaneously				
10	+31	1·76	0·73	38·2
20	+20	1·62	0·72	38·2
30	+19	1·58	0·80	36·0
40	+17	1·58	0·72	34·7
50	+ 7	1·36	0·78	30·5
80	+124	2·41	0·71	56·9*

* Symptoms of hypoglycaemia.

Exp. 5 (*b*). Same subject as preceding (5 *a*). Respiratory metabolism after subcutaneous injection of 1 cc. of pituitrin.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.
0	−10	1·21	0·71	25·1
Pituitrin subcutaneously (injection painful)				
10	− 6	1·27	0·72	26·1
20	−19	1·06	0·81	25·0
30	−19	1·09	0·74	25·1
40	−12	1·17	0·77	25·2
70	−13	1·16	0·76	26·7

Exp. 5 (*c*). Same subject as in two preceding experiments (5 *a* and 5 *b*). Respiratory metabolism after simultaneous injection of insulin (10 units) and pituitrin (1 cc.) subcutaneously.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.
0	− 2	1·33	0·72	38·5
Insulin and pituitrin subcutaneously				
10	− 5	1·28	0·74	38·5
20	− 2	1·35	0·70	39·5
30	+17	1·58	0·71	40·0
40	+ 1	1·31	0·89	42·5
70	− 9	1·21	0·77	37·8

Exp. 6 (*a*). Respiratory metabolism in normal male after subcutaneous injection of 15 units of insulin.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	−3	1·41	0·73	34·75	0·104	2·906
Insulin 15 units subcutaneously						
10	−6	1·36	0·72	31·72	0·104	2·777
20	+5	1·51	0·74	36·5	0·074	2·765
30	−2	1·41	0·77	37·6	0·066	2·687
40	−1	1·42	0·73	38·5	—	2·705

Exp. 6 (*b*). Same subject as preceding (6 *a*). Respiratory metabolism in normal male after 1 cc. pituitrin subcutaneously.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.
0	−3	1·32	0·81	34·5
Pituitrin subcutaneously				
15	−5	1·27	0·77	35·0
25	+5	1·386	0·86	36·0
35	+1	1·380	0·73	34·5

Exp. 6 (*c*). Same subject as preceding experiments (6 *a* and 6 *b*). Respiratory metabolism, blood-sugar and inorganic blood-phosphate in normal male after subcutaneous injection of insulin, 15 units, followed 7 mins. later by pituitrin 1 cc.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	+6	1·61	0·74	40·75	0·111	2·928
Insulin subcutaneously						
6	+11	1·7	0·79	42·25	0·111	2·875
7	Pituitrin subcutaneously					
22	+4	1·5	0·96	46·25	0·100	2·823
30	+11	1·66	0·85	45·5	0·104	2·703
50	−14	1·25	1·1	43·5	0·111	—
65	−18	1·42	1·0	40·75	0·111	2·652

Exp. 6 (*d*). Same as preceding but pituitrin given 25 mins. after insulin. Respiratory metabolism.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.
0	−16	1·19	0·81	29·5
Insulin subcutaneously				
10	−14	1·26	0·78	28·5
25	Pituitrin subcutaneously			
35	−13	1·21	0·86	33
45	−5	1·32	0·86	39·5
55	−9	1·27	0·85	34
65	−11	1·25	0·84	32

Exp. 6 (*e*). Respiratory metabolism, blood-sugar and inorganic blood-phosphate in normal male (different subject from preceding) after insulin, 15 units, followed 62 mins. later by pituitrin 1 cc.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	+11	1·76	0·76	31	0·100	3·515
Insulin subcutaneously						
30	+30	2·06	0·78	37·5	0·082	2·928
60	+24	1·17	0·76	36	0·076	2·510
62	Pituitrin subcutaneously					
64	+30	2·01	0·86	42·35	0·076	2·130
74	+32	2·07	0·80	43·25	0·079	2·162
84	+18	1·85	0·81	36·2	0·082	2·381
94	+26	2·01	0·75	37·52	0·086	2·343
104	+11	1·75	0·77	33·75	0·086	2·421

Exp. 7 (*a*). Respiratory metabolism, blood-sugar and inorganic blood-phosphate in normal subject after subcutaneous injection of 0·75 cc. of adrenaline (1 in 1000).

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	+ 8	1·99	0·81	40·5	0·091	3·652
Adrenaline subcutaneously						
10	+35	2·41	0·94	57·0	0·117	3·255
20	+40	2·59	0·80	54·25	0·133	3·181
30	+45	2·69	0·78	53·0	0·133	3·232
40	− 7	1·69	0·93	41·25	0·25	3·308

Exp. 7 (*b*). Respiratory metabolism in normal male after 0·75 cc. of adrenaline (1 in 1000) subcutaneously.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.
0	−13	1·39	0·76	42
Adrenaline subcutaneously				
10	+ 8	1·62	0·84	53·7
20	+ 8	1·6	0·82	50·25
30	− 5	1·5	0·76	47
40	− 8	1·34	0·86	49
50	− 9	1·39	0·75	43

Exp. 7 (*c*). Same as 7 (*b*). Dextrose 20 g. injected intravenously and adrenaline 0·75 cc. subcutaneously.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.
0	−27	1·3	0·72	38
Dextrose intravenously; adrenaline subcutaneously				
10	+42	2·15	0·82	72
20	+ 7	1·63	0·78	54
30	− 4	1·46	0·80	48
40	−18	1·25	0·76	38
50	−16	1·28	0·78	40

Exp. 8. Respiratory metabolism, blood-sugar and inorganic blood-phosphate in a patient suffering from exophthalmic goitre, after oral administration of 50 g. of dextrose.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	+ 52	1·58	0·85	46·75	0·075	3·947
Dextrose orally						
30	+102	2·17	0·81	51·25	0·145	3·448
60	+ 48	1·88	0·71	58·0	0·205	3·355
90	+ 52	1·86	0·85	54·5	0·162	3·488
120	+ 37	1·71	0·78	57·5	—	—

B. Animal experiments.

Exp. 1. Rabbit 2340 g. Effect of pituitrin alone on blood-sugar and inorganic blood-phosphate.

Time mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	0·124	3·472
Pituitrin 0·5 cc. intravenously		
15	0·159	3·874
35	0·124	3·204

Exp. 2 (*a*). Rabbit 2950 g. Effect of dihydroxyacetone alone.

Time mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	0·145	4·166
Dihydroxyacetone 2·5 g. intravenously		
15	0·288	3·424
30	0·247	3·100
45	0·236	2·976

Exp. 2 (*b*). Same animal as Exp. 2 (*a*). Effect of pituitrin and dihydroxyacetone together.

Time mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	0·111	2·702
Pituitrin 0·5 cc. + dihydroxyacetone 2·5 g. intravenously.		
15	0·255	3·120
30	0·238	3·030
45	0·217	2·630

Exp. 3 (*a*). Rabbit 2500 g. Effect of dextrose alone.

Time mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	0·97	3·900
Dextrose 2·5 g. intravenously		
15	0·186	3·676
30	0·115	3·472
45	0·084	3·378

Exp. 3 (*b*). Same animal. Effect of dextrose and pituitrin together.

Time mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	0·132	3·570
Pituitrin 0·5 cc. + dextrose 2·5 g. intravenously		
15	0·302	3·56
30	0·164	3·444

Exp. 4 (*a*). Rabbit 1390 g. Effect of dihydroxyacetone and pituitrin together, using smaller dose of pituitrin than in preceding experiments

Time mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	0·113	3·387
Pituitrin 0·1 cc. + dihydroxyacetone 1·39 g. intravenously		
20	0·294	2·876
45	0·168	2·763

Exp. 4 (*b*). Same animal as Exp. 3 (*a*). Effect of dextrose and pituitrin together in similar doses.

Time mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	0·120	3·402
Pituitrin 0·1 cc. + dextrose 1·39 g. intravenously		
20	0·175	3·735
45	0·121	3·206

Exp. 5 (*a*). Rabbit 3000 g. Effect of dihydroxyacetone and pituitrin together.

Time mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	0·125	3·786
Pituitrin 0·1 cc. + dihydroxyacetone 2·5 g. intravenously		
15	0·264	3·204
30	0·187	3·110

Exp. 5 (*b*). Same animal. Effect of dextrose and pituitrin together in similar doses.

Time mins.	Blood-sugar g. per 100 cc.	Inorganic blood-phosphate mg. per 100 cc.
0	0·115	3·2110
Pituitrin 0·1 cc. + dextrose 2·5 g. intravenously		
15	0·285	3·7668
30	0·135	3·1865

Discussion.

The above experiments appear to show that pituitrin has a marked inhibitory action upon the metabolism of carbohydrates. Thus Exp. 1 (*a*) shows the typical changes which occur in the respiratory metabolism and blood-sugar in the healthy individual following the administration of 20 g. of dextrose intravenously. There is first a gradual rise in the heat production and oxygen consumption, the R.Q. and the respiratory volume, maximal values being attained in approximately 20–30 minutes. (The high initial R.Q. in this experiment may have been due to overbreathing.) The blood-sugar following the injection falls rapidly, reaching the base line in 30–40 minutes. When the same amount of dextrose was administered to this individual along with pituitrin, injected subcutaneously, there was little if any rise in the heat production, oxygen consumption, R.Q. or respiratory volume, by the time these would have been at their height in the control. In fact, save for an initial increase in the metabolic rate and oxygen consumption, for which changes in blood flow resulting from the injection of fluid may have been responsible, there was remarkably little alteration in the metabolism for the first 30 or 40 minutes. After this, however, coinciding with the passing off of the vaso-constriction, as evidenced by diminution in the pallor of the skin, there was a rise in all these values with the exception of the R.Q. which fell a little when the heat production and oxygen consumption were at a maximum. The R.Q. rose to its highest at a later period. It is interesting to note that the blood-sugar fell very slowly during the period when the respiratory metabolism showed little change, being still well above the base line at the end of 40 minutes. After this, however, it fell while the metabolism rose.

In Exp. 2, which was performed upon a diabetic patient on a low diet, the changes are less striking but are of a similar character. The R.Q., which was remarkably low, here attained its maximum after all the other values had fallen.

The effect of pituitrin upon the changes in respiratory metabolism which follow the intravenous administration of dihydroxyacetone is equally striking. In spite of the administration of a dose of pituitrin sufficient almost completely to annul the rise in metabolism due to dextrose during the first 20 minutes of the experiment, there was still a considerable rise in the metabolism during this period; in fact the actual rise was even greater than that obtained with dextrose alone. Nevertheless, the rise in metabolism was not nearly as great as with dihydroxyacetone alone, the percentage inhibition due to pituitrin being just as great as in the case of dextrose. In addition to the inhibition of the metabolism, however, the form of the curves for heat production and oxygen consumption were entirely altered, resembling in certain respects those obtained with dextrose alone. Thus, whereas dihydroxyacetone alone caused an immediate rise in metabolism with maximal values

soon after the completion of the injection, followed by a rapid fall to almost normal at the end of 30–40 minutes, pituitrin given along with dihydroxyacetone inhibited the initial acceleration in metabolism (see 10 minutes' sample), there being instead a gradual rise reaching a maximum in 30 minutes. The R.Q., however, varied inversely with the oxygen consumption and heat production during this period. A slight secondary rise in the metabolic rate, oxygen consumption, R.Q. and respiratory volume was observed at the end of 70 minutes.

In the experiments in which insulin and pituitrin were injected together, the results were not nearly so clear cut as when pituitrin was given along with carbohydrate, owing to the changes in respiratory metabolism produced by either hormone during the first 30–40 minutes after their administration being so slight as to be of doubtful significance. McKinlay [1921], Bowman and Grabfield [1926] and Castex and Schteingart [1926] report a rise in metabolism of about 10 % occurring 20–30 minutes after the administration of pituitrin alone; but our results are not so uniform. Thus, in Exp. 4 (*b*) there was a slight rise in the heat production, oxygen consumption and R.Q.; in Exp. 6 (*b*) there was a preliminary fall during the first 15 minutes, followed by a rise, while in Exp. 5 (*b*) there was a more prolonged fall in the heat production and oxygen consumption, but not in the R.Q. The slight rise in metabolism obtained in the 10 minutes' sample in this experiment may have been occasioned by the pain of the injection.

These irregular results may be due to pituitrin on the one hand stimulating intestinal peristalsis, which would tend to increase the oxygen consumption, and on the other hand depressing the metabolism in other tissues. The effect upon the respiratory metabolism would depend upon the time relationship and relative potency of these two factors. In both Exps. 5 (*c*) and 6, (*c*) in which insulin and pituitrin were injected either simultaneously or within a comparatively short interval of one another, pituitrin seemed to inhibit the rise in metabolism or cause a fall at the time of the corresponding observations in the control with insulin alone. Since, however, pituitrin by itself caused a fall in metabolism at these times in this particular series of experiments, the effect of the administration of the two hormones together was apparently such as might have resulted from a combination of their independent actions.

When pituitrin was injected at a longer interval (half an hour to an hour) after insulin (Exps. 6 (*d*) and 6 (*e*)) a slight rise in the heat production and R.Q. was observed. This may possibly be connected with the liberation of glucose from the liver or with the contractions of the stomach and intestine which are more readily excited in hypoglycaemia. (Hunger pains are common during hypoglycaemia.)

Our findings regarding the effect of pituitrin upon the blood-sugar are in agreement with those of other observers, namely, a delay in the disappearance of injected dextrose from the blood [Hines, Leese and Boyd, 1927; Achard, Ribot and Binet, 1919], inhibition of hypoglycaemia or a return of the blood-

sugar towards the normal level in hypoglycaemic animals after insulin [cf. Burn, 1923; Olmstead and Logan, 1923; Moehlig and Ainslee, 1925; Lawrence and Hewlett, 1925; Geiling and Britton, 1927], while injection of pituitrin alone caused a transient but inconstant rise in the blood-sugar during the first 30 minutes after its administration [cf. Borchardt, 1908; Goetsch, Cushing and Jackson, 1911; Parton and Katz-Klein, 1921; Lindlau, 1928; Blotner and Fitz, 1928].

With regard to phosphate metabolism, pituitrin alone in the doses employed in the human subject caused a slight fall in the percentage of inorganic phosphate in the blood, but given in relatively large doses to rabbits, it caused a marked but transient rise. When administered to either human beings or rabbits with hypoglycaemia and a low inorganic blood-phosphate resulting from insulin administration, it caused the percentage of inorganic blood-phosphate to rise. Similar results were obtained with adrenaline as with pituitrin.

The results so far reviewed, taken as a whole, might at first sight seem to bear out the contention that pituitrin inactivates insulin, thereby bringing about, temporarily, a state of affairs analogous to that obtaining in diabetes. Hyperglycaemia, delayed disappearance from the blood of injected carbohydrate, diminished response in respiratory metabolism following administration of carbohydrate and an increased percentage in inorganic blood-phosphate, all of which may follow the injection of suitable doses of pituitrin, are also features characteristic of the diabetic condition. Taken in conjunction with the observation that pituitrin seems to inhibit any slight rise in metabolism following administration of insulin, the evidence in support of a direct antagonism would at first sight appear to be strong and to have the advantage of economy of hypothesis. Nevertheless, in certain respects this explanation does not appear to be wholly satisfactory. Thus, pituitrin, except in the large doses employed in rabbits, did not, when given alone, cause any rise in the inorganic blood-phosphate, but rather a slight fall; moreover, in spite of being given in doses sufficient completely to prevent the fall in blood-sugar following insulin administration in the human subject, it did not prevent the inorganic blood-phosphate from falling (Exp. 6 (*c*)), and, although the fall may have been temporarily checked, the blood-sugar remained at the normal level while the level of inorganic blood-phosphate continued to decline.

Again, pituitrin caused no definite fall in the respiratory quotient, but more often a rise.

Further, in the experiment with dihydroxyacetone and pituitrin (Exp. 3 (*b*)), the curves of metabolism fail to reproduce the features of those obtained in diabetes. In the latter [cf. Lambie and Redhead, 1927] the heat production, oxygen consumption and R.Q. attained their maxima together soon after the injection, whereas in the experiment referred to the heat production and oxygen consumption gradually rose, reaching their height at the end of 30 minutes, while the respiratory quotient was at its highest in 10 minutes and declined *pari passu* with the rise in the metabolic rate.

In view of these differences between the effects of deficiency of insulin and the alleged inactivation of insulin by pituitrin, it is necessary to consider other possible explanations of the phenomena. Krogh [1922] has shown that pituitrin not only constricts the arterioles, but markedly increases the contractile tonus of capillaries, the effect of which would be to diminish the capillary bed. This would lead to a decreased rate of diffusion of injected material out of the blood into the tissues in which it was to be metabolised and in this way it may be possible to explain the delay in the rise in metabolism with dihydroxyacetone or dextrose, and the delay in the disappearance of sugar from the blood after intravenous injection. The occasional sudden rise in metabolism during and immediately after intravenous injection may have been the result of a temporary increase of blood flow due to the volume of fluid injected, especially since the fluid was hypertonic. The experiments of Hines, Leese and Boyd [1927] did not show any definite change in the heat production or R.Q. as a result of injecting pituitrin during continuous transfusion of dextrose, but this may have been due partly to the very large quantity of dextrose injected and to the fact that there was a continuous transfusion of fluid into the circulation. Their results are therefore not inconsistent with our own, but are due to the different conditions of the experiments.

The depression in metabolism sometimes observed following injection of pituitrin alone may again be due to diminished blood flow through the muscles, while the subsequent rise may perhaps be related to the contraction of non-striped muscle, particularly that of the intestine (borborygmi being frequently audible) and to increased work of the heart.

The rise in inorganic blood-phosphate noted after injection of large doses of pituitrin might also be explainable as a result of local asphyxia in the muscle cells, while increase in the blood-sugar may perhaps be related to changes in the portal circulation [cf. Clark, 1928].

It appears reasonable to expect that drugs, which, like pituitrin and adrenaline, alter the blood flow through organs, particularly those which, like muscle, are important seats of heat production, should be capable of modifying metabolism, and, if at the same time they cause change in the tonus of muscle or the work of the heart, further complications are to be anticipated. The metabolic changes which result from the administration of adrenaline should therefore be considered in this light also, but further experiment is necessary before definite conclusions can be drawn. Krogh [1922] points out that adrenaline differs from pituitrin in having a more powerful action on the arterioles than upon the capillaries, but all arterioles in the body are not equally affected. Hoskins, Gunning and Berry [1916] and Gunning [1917] have brought forward evidence to show that adrenaline in small doses causes blood to be diverted from the splanchnic area and skin while the blood flow through the voluntary muscles and myocardium is increased. This, together with the increased work of the heart, may be responsible for the

marked and sudden rise in metabolism following the administration of adrenaline alone, but it remains an open question as to how the metabolic processes in and the blood flow through the muscles are causally related.

Our experiments do not support the view that the rise in metabolism is to be ascribed to the hyperglycaemia. In the first place, the rise in metabolism following injection of adrenaline is very great even with a slight hyperglycaemia, whereas with a much greater hyperglycaemia produced by intravenous injection of dextrose there was less increase in the metabolism [cf. Boothby and Sandiford, 1921]. The time relationship between hyperglycaemia and the change in respiratory metabolism is also quite different in the two cases. Moreover, the rise in metabolism which occurs after injecting adrenaline is much more rapid than after intravenous injection of dextrose. Thus, 10 minutes after administration of adrenaline in Exps. 7 (*a*) and 7 (*b*) there was a 20 % rise in the metabolism at a time when the blood-sugar had risen (cf. Exp. 7 (*a*)) only slightly above the fasting level, whereas 10 minutes after injection of 20 g. of dextrose intravenously (Exp. 1 (*a*)) there was practically no increase in respiratory metabolism although there was a definite hyperglycaemia. It may be suggested as a possible explanation of these results that the dextrose liberated by the liver is more immediately available for combustion than ordinary dextrose, but apart from the lack of evidence to support this view, there is the observation that in Exp. 7 (*a*) with adrenaline the blood-sugar continued to rise even when the metabolic rate was falling. There appears, therefore, to be no high degree of correlation between hyperglycaemia and heightened metabolism after administration of adrenaline.

As to the method by which adrenaline causes hyperglycaemia, stimulation of the endings of the splanchnic nerves in the liver, changes in the portal circulation [cf. Clark, 1928], and perhaps the breaking down of muscle-glycogen, may all play a part; but there is no need to assume a direct antagonism to insulin.

The influence of adrenaline upon phosphorus metabolism has been investigated by Allan, Dickson and Markowitz [1924] and the results obtained were similar to those produced by insulin, indicating that, as in the case of pituitrin, there was no antagonism between the two in this respect.

The effect of simultaneous injection of adrenaline and insulin upon the respiratory metabolism is such as would be expected to result from the combined effect of each separately. Thus, in Exp. 7 (*b*), the percentage rise in calorie consumption 20 and 30 minutes after adrenaline alone was 21 % and 8 % respectively. In most experiments with 20 g. of dextrose alone, intravenously, the percentage rise at these periods is 5 % and 10 % respectively. The combined effects of dextrose and adrenaline would therefore give + 26 % and + 18 %. In Exp. 7 (*c*) with both adrenaline and dextrose together the actual figures obtained were + 34 % and + 23 %; there is, therefore, no indication of antagonism here.

As in the case of adrenaline, no evidence could be obtained of any

inhibitory effect of excessive thyroid activity upon the metabolism of dextrose, but rather the reverse. Thus, in Exp. 8 the metabolic rate rose to over 100 % 30 minutes after the administration of 50 g. of dextrose by the mouth to a patient with exophthalmic goitre.

The facts so far brought forward, therefore, afford no definite proof of there being a direct inactivation of one hormone by the other, and, until the changes in the circulation can be excluded as the cause of the apparent antagonism, it appears unnecessary to assume that any more direct and specific action is involved. It is true that in certain forms of pituitary disease (acromegaly) there is often diminished sugar tolerance, or even a condition indistinguishable from true diabetes, together with marked resistance to insulin [Ulrich, 1928], but no obvious disturbance of the circulation is present in such cases. The assumption that the low carbohydrate tolerance is due to excessive activity of the posterior lobe of the pituitary body is, however, unwarranted, since the glycosuria occurs in connection with a definite type of tumour of the anterior lobe, namely, the eosinophil adenoma, and not with other tumours, although the latter are more numerous [cf. Davidoff and Cushing, 1927]. Although nothing is at present known regarding the effect of repeated small doses of pituitrin administered over a long period, the fact that the apparent inhibition of carbohydrate metabolism has so far only been obtained with doses sufficiently large to cause obvious circulatory disturbances (*e.g.* blanching in man), makes the resemblance to the clinical pituitary glycosuria incomplete. Since it has been impossible to settle the question of the nature of the antagonism between pituitrin and insulin the significance to be attached to the peculiar behaviour of dihydroxyacetone in the above experiment must likewise remain an open question. The fact that it caused a greater rise in metabolism than dextrose, and brought about a fall in inorganic blood-phosphate in rabbits when dextrose failed to do so after the administration of pituitrin, is merely in conformity with previous findings [Lambie and Redhead, 1927].

Summary.

1. In the human subject pituitrin frequently causes a transient fall in metabolism followed by a rise. Sometimes only a rise is observed. In small doses it causes a slight fall in the inorganic phosphate of the blood.

2. Pituitrin apparently inhibits the slight rise in metabolism which occurs after the administration of insulin; but the changes produced by either insulin or pituitrin are not sufficiently marked to be of much significance.

3. Pituitrin delays and reduces the rise in metabolism and the fall in blood-sugar following the intravenous administration of dextrose or dihydroxyacetone.

4. Pituitrin in small doses sufficient to prevent the fall in blood-sugar resulting from administration of 15 units of insulin did not prevent the fall in inorganic blood-phosphate.

5. Pituitrin administered in large doses to rabbits causes a transitory rise in the inorganic blood-phosphate and for a short period inhibits the fall in inorganic phosphate resulting from administration of dextrose or dihydroxyacetone. A dose of pituitrin which just suffices to prevent the fall in inorganic phosphate following administration of dextrose may fail to overcome the fall due to dihydroxyacetone.

6. In animals rendered hypoglycaemic as a result of insulin administration pituitrin causes the blood-sugar and inorganic blood-phosphate to return to the normal level or even to exceed it.

7. Adrenaline administered alone causes a marked and rapid rise in metabolism which is not solely dependent upon the rise in blood-sugar.

8. Adrenaline does not inhibit the rise in metabolism following intravenous administration of dextrose.

9. Adrenaline administered in large doses to hypoglycaemic animals causes a rise in inorganic blood-phosphate.

10. The question is discussed as to how far the effects of pituitrin and adrenaline upon metabolism result from the changes in the circulation and distribution of blood in the body or whether some more direct and specific antagonism to insulin is to be assumed. It is concluded that experiments so far recorded have not proved unequivocally that such a direct action exists.

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LXXI. THE REACTION OF AZINE COMPOUNDS WITH PROTEOLYTIC ENZYMES.

By GEORGE MAXWELL RICHARDSON
(1851 *Exhibition Research Scholar*)
and ROBERT KEITH CANNAN.

From the Department of Physiology and Biochemistry, University College, London.

(*Received June 18th, 1929.*)

The formation of precipitates when various azine dyes are added to solutions of proteolytic enzymes has been remarked by a number of observers. In some cases, moreover, it has been shown that the mother liquors have lost their protease activity and that this has, in part, been transferred to the precipitates [Holzberg, 1913; Wood, 1918; Marston, 1923; Forbes, 1927]. The observations and conclusions of Marston are of particular interest. Marston found that safranine was a specific precipitant for all the protease activities he tested—pepsin, trypsin, erepsin, yeast protease, papain. The dye did not, however, remove other non-proteolytic enzymes from solution. Since, moreover, other azonium salts, azines, and eurhodines behaved similarly towards trypsin, Marston concluded that he was observing a specific interaction between the azine nucleus and the structure peculiar to a protease. He further fortified this argument by drawing an analogy between the azine ring as represented by the various dyes employed and the piperazine ring which he believes to be a dominant feature of the protein molecule. Robertson [1928], indeed, has employed Marston's argument to support the diketopiperazine structure of protein. Now the view that the azine nucleus found in these dyes resembles the heterocyclic ring of a diketopiperazine is open to serious criticism upon structural grounds and makes the acceptance of the full implications of Marston's argument difficult. On the other hand, there is no doubt that valuable opportunities are opened by the demonstration that a simple well-defined structure reacts with a group of enzymes in a specific way which parallels the catalytic specificity of that group of enzymes.

In particular, Marston's observations suggested to us an opportunity for the examination of the kinetics of protease activity by means of a quantitative study of the anticatalytic effect of azine compounds on the activity of the protease. At the same time it was hoped to assemble data which would permit the precise use of this reaction as an economical means of purifying protease preparations.

The poisoning of protease activity by azine dyes.

Protease activity was determined by the viscosity method of Northrop and Hussey [1923], where the measure of activity is the time taken to produce a definite degree of viscosity change in a solution of gelatin. Results were conveniently reproducible and a relative measure of activity accurate to within 10 % was obtained when all the necessary precautions were observed. The comparison of activities was most reliable if the different enzyme preparations were separately diluted to produce approximately similar rates of viscosity change. A dilution which produced half of the total viscosity change in 10 to 30 minutes was adopted as being most convenient. Comparison of activities determined on substrate samples of markedly different initial viscosity is not strictly valid, but is useful qualitatively.

Viscosity determinations at 30° were made in viscosimeters standardised against distilled water and, after each determination, cleaned with chromic acid overnight. The substrate solutions were 2·75 % solutions of isoelectric gelatin [Loeb, 1922] adjusted with phosphoric acid or phosphate to a p_H of 2·7 for pepsin and 7·5 for trypsin determinations. After addition of a trace of thymol, these bulk solutions were stored in small bottles in the refrigerator. For use each bottle was warmed to melt the gelatin and maintained at 30° for at least 2 hours. Then, as required, samples were removed, mixed at zero time with one-twentieth volume of the enzyme preparation, and pipetted in 7 cc. amounts to the viscosimeters, every precaution being taken to minimise cooling of the solutions below 30°. Initially, determinations were made in duplicate until the precaution was found unnecessary.

Table I. *Poisoning of protease activity by azine dyes.*

Enzyme	Azine dye	$[E]$	$[D]$	t mins.	Gelatin sample
Dog's gastric juice	Safranine	0·002	—	26	a
		0·002	0·01	27	a
		0·002	0·05	37	a
		0·002	0·2	69	a
		0·002	0·6	100	b
Benger's trypsin	Neutral red	0·0015	—	29	c
		0·0015	0·00025	26	c
		0·0015	0·0012	26	c
		0·0015	0·0063	25	c
	Safranine	0·0015	0·0063	25	c
		0·0015	—	44	d
		0·0015	0·0063	43	d
		0·0015	0·025	39	d
		0·0015	0·1	47	d
		0·0015	0·2	70	d
		0·0015	0·6	90	d

$[E]$ = concentration of enzyme in 100 cc. of the digestion mixture in terms of total solids (g.) of the original preparation.

$[D]$ = concentration of dye (g. %) in the digestion mixture.

t = time, corrected to a constant arbitrary enzyme concentration, for 50 % viscosity change in the gelatin.

p_H for pepsin experiments 2·7, for trypsin experiments 7·5.

In Table I and Fig. 1 are summarised the results of experiments on gastric juice from a dog's Pavlov fistula (0·42 % solids) and upon a highly active

pancreatic extract (6·1 % solids) kindly prepared for us by Benger's Food Products Ltd.

It is obvious that dye concentrations of even 0·05 to 0·1 % in no way retard the hydrolysis of the gelatin by the enzyme. Moreover, it should be pointed out that the concentration of dye at which retardation does definitely occur is the critical concentration for immediate precipitation of the enzyme complex (though precipitation occurs in 24 hours with all dye concentrations above 0·006 %). Therefore, not only is there no poisoning with small concentrations of dye, but such diminution in activity as is observed with high concentrations occurs only upon visible separation of a gelatinous precipitate in the viscosimeter. The artificial effect of such a precipitate is obvious, and appears in the rather erratic viscosity curves at these concentrations (Fig. 1). Hence the indicated decrease in activity may well be merely apparent.

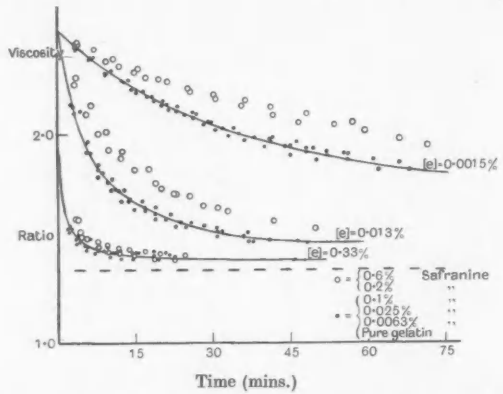

Fig. 1. Poisoning test (safranine on Benger's trypsin) $p_H = 7·5$.

To test this point the diastase activity of the Benger's pancreas extract was investigated with starch in the presence of varying concentrations of dye. Though results were more erratic, an effect running quite parallel to the above was obtained, so that, since diastase activity is not precipitated by azine dyes [Marston, 1923], any retardation would appear to be artificial and dependent at least predominantly on the gelatinous nature of the precipitate. Complete confirmation of this view was received, at least for the trypsin experiments, when it was discovered later that the tryptic activity of the Benger's extract is not significantly precipitated by azine dyes.

These results do not deny a specific chemical reaction between the dye and the catalytically active grouping of the protease. They do suggest, however, that the hypothetical complex formed is so largely dissociated in solution that the active mass of free enzyme is unaffected by such concentrations of dye as will cause an ultimate separation of the insoluble complex.

Further information was sought by direct studies of the protease activities of azine precipitates and of the mother liquors from which these precipitates have been separated.

The precipitation of pepsin and trypsin by neutral red.

The factor which has limited former studies of these precipitates has been the difficulty of redissolving the active material. Only in the case of pepsin had this been found possible [Marston, 1923; Forbes, 1927], advantage being taken of the fact that in dilute solutions of strong acids the precipitate redissolves and the dye may be extracted by such solvents as amyl alcohol. Even in this case, however, emulsification during extraction rendered quantitative recovery of the enzyme difficult.

Now Marston found that the leuco-azine bases do not form precipitates with enzymes. We find further that a reducing agent such as sodium hyposulphite will redissolve an existing precipitate. Moreover, the reducing agent does not destroy the activity of the protease present. A simple method of regaining the precipitated enzyme in solution at once suggests itself. The precipitate is removed from the reaction mixture by centrifuging, washed with 0·1 % safranine and suspended in an appropriate buffer. Sodium hyposulphite is added and the leuco-base extracted with benzene in a vessel protected from oxygen. A useful extraction device which avoids any emulsification consists of a shallow cylinder in which a thin layer of redissolved precipitate covered with a layer of benzene is gently and slowly rocked. Two changes of solvent give efficient extraction, the whole process being complete in an hour.

A still simpler method of extracting the precipitate—in the case of trypsin—is based upon the observation that a neutral red precipitate will redissolve in solutions of p_H more alkaline than about 7, *i.e.* when the basic dissociation of neutral red is suppressed. It is necessary therefore only to precipitate the preparation at p_H 6, adjust the solution to p_H 9, and extract the yellow dye with benzene. The same rocking device may be employed.

The various reagents and manipulations involved in these methods have no destructive effect upon either pepsin or trypsin. This was established by comparing the activity of an enzyme solution before a precipitate was formed in it by addition of dye and after the precipitate had been redissolved in its own mother liquors and extracted by one of the methods described.

In estimating the activity remaining in the mother liquors it proved to be unnecessary to carry out a preliminary extraction of the dye since the further degree of dilution involved in carrying out the activity test reduced the concentration of dye far below that which had been found to give significant "poisoning" (Table I).

Protease activity of the enzyme precipitates and supernatant liquors.

In these investigations the enzyme solutions were buffered at p_H 3·9 (acetate buffer) for pepsin and at p_H 6·0 (phosphate buffer) for trypsin. p_H determinations were made with a Hildebrand hydrogen electrode slightly modified to eliminate frothing. Samples of the buffered preparations were then mixed with varying concentrations of dye, allowed to flocculate for 15 hours in the refrigerator, centrifuged for 20 minutes in a high speed centrifuge, and the supernatant liquors decanted from the precipitate. The precipitate was dried to constant weight in a steam-oven, and the extent of precipitation of the protease activity was estimated by the loss of activity in the supernatant liquors. In this way the loss of activity was compared with the amount of precipitate carrying it down and with the concentration of the precipitating dye. The data appear in Table II.

Table II. *Protease activity of the supernatant liquors.*

Enzyme	Dye	$[D]$	$\frac{100w}{S}$	t (supernatant) mins.	Gelatin sample
Harrington's pepsin 2 % solution	Neutral red	—	Not determined	11	e
		0·024	,,	50	e
		0·045	,,	80	e
		0·083	,,	150	e
Dog's gastric juice $[E]=0·195$	Safranine	—	—	6	f
		0·005	—	6·5	f
		0·01	9	8	f
		0·02	10	16	f
		0·05	19·5	144	f
		0·1	23·5	550	f
		0·2	24	700	f
		0·5	Not determined	800	f
Harrington's trypsin 3 % solution	Safranine	—	,,	7	g
		1·0	,,	10	g
Benger's trypsin $[E]=2·6$	Safranine	—	—	29	h
		0·1	5	28	h
		0·5	12	29	h
		1·0	17	28	h

$[E]$ = concentration of enzyme in 100 cc. of the precipitation mixture in terms of total solids (g.) of the original preparation.

$[D]$ = concentration of dye (g. %) in the precipitation mixture.

$\frac{100w}{S}$ = dry weight of precipitate expressed as percentage of the total solids of the original preparation.

$[E]$ and $[D]$ are diluted 20 times (or 2000 times for Benger's trypsin) in the activity tests.

A saturated aqueous solution of safranine contains just above 1 % of dye.

The data for pepsin precipitation are in general agreement with Marston's views, since loss of activity runs parallel with both the concentration of dye and the weight of precipitate. However, it is noticed that, though the critical dye concentration for removal of peptic activity from solution is 0·02 to 0·1 % with practically complete removal at 0·2 %, yet by weight 38 % of the precipitate at 0·2 % has already been precipitated in a dye concentration of

only 0·01 %. This anomaly becomes more significant when considered in the light of the trypsin results, for here we find the quite unexpected fact that, though the weight of the precipitate varies definitely with the dye concentration, the activity of the supernatant liquor is not measurably reduced by loss of this precipitate. The activity of such precipitates was quite definite, but, as Table III indicates, it varied with the origin of the preparation, and was quite a negligible fraction of the total activity.

Now Marston claims to have found 64 % of the total activity of a trypsin solution in his precipitate. This variability in extent of trypsin precipitation allows of no simple explanation other than the obvious one that the precipitation does not specifically involve the enzyme. We incline to the view that trypsin preparations may or may not contain varying amounts of a substance (probably protein) with which the enzyme tends to be closely and specifically associated, and that this substance is precipitated by azine dyes removing the enzyme with it. It is of interest, therefore, that Wood [1918] reports an active preparation of trypsin which failed to give any precipitate with safranine.

Attempts were now made to encourage removal of the dye by inducing formation of some foreign precipitate in the solution. Robertson mentions the precipitation of phosphates and nucleic acid by azine dyes, so experiments were conducted in which Benger's trypsin was mixed with varying concentrations of nucleic acid and then precipitated in the usual fashion. The results were entirely negative and appear in Table III. Precipitations were performed in 0·1 % neutral red at p_H 6. Activities were determined in the supernatant liquors after extraction of the dye with benzene at p_H 9.

Table III.

(*a*) *Protease activity of the precipitates.*

(*b*) *Effect of induced precipitation.*

$[D]=0{\cdot}1$ %.

Enzyme	Nucleic acid conc. %	t mins.
10 % Harrington's trypsin	—	1·5
,, ,, extracted precipitate	—	36
Benger's trypsin, 3 % total solids	—	15
,, etc., extracted precipitate	—	6×10^4
,, ,, extracted supernatant	—	12
,, ,, ,,	0·016	13
,, ,, ,,	0·08	11·5
,, ,, ,,	0·4	12·5

The values of t given for the extracted precipitates have been corrected so as to define the activity of these extracts when diluted to the same volume as that of the enzyme solution from which the precipitates were obtained. In the case of Benger's trypsin this leads to a calculation value which has only qualitative significance indicating virtual absence of activity in the precipitate.

It is obvious that no trypsin activity has been incorporated within the bulky precipitate formed between neutral red and nucleic acid. It does not appear therefore that the presence of nucleic acid in the enzyme preparations of Marston was responsible for his results.

Effect of p_H *on precipitation of pepsin.*

Aliquots of a sample of mixed gastric juice were titrated to definite p_H values with a citric acid-citrate buffer of p_H 6, being at the same time adjusted to equal dilution. Safranine (p_H 2·6) was added, the final concentration being 0·2 % dye and 0·175 % pepsin (total solids). The solutions were left for 15 hours for flocculation, the precipitates separated in the centrifuge, and the mother liquors decanted and tested for activity. The dry weights of the precipitates were determined. The results appear in Fig. 2 and in Table IV.

Table IV. *Effect of* p_H *on precipitation of gastric juice by safranine.*

$[D]=0{\cdot}2$ %. $[E]=0{\cdot}175$ %.

p_H	t mins.	$\frac{100T}{t}$	$\frac{100w}{S}$	$100\left(1-\frac{T}{t}\right)$
1·0 (dye-free)	7·5	100	—	—
2·45 (dye-free)	8	95	—	—
4·0 (dye-free)	5·5	135	—	—
1·0	11	68	16	32
1·95	14·5	52	18	48
2·2	18	42	19	58
2·45	30	25	21	75
2·75	60	12·5	22	87·5
2·95	160	4·7	27	95·3
3·97	1300	0·6	65	99·4
4·85	1300	0·6	91	99·4
(From Table II)				
3·9	700 (× 1·1)	0·9	24	99·1
3·9 (dye-free)	6 (× 1·1)	109	—	—

$\frac{100T}{t}$ = the percentage of the original protease activity remaining in the supernatant liquors after precipitation at the given p_H ($T=7{\cdot}5$).

$100\left(1-\frac{T}{t}\right)$ = the percentage of the original protease activity removed with the precipitate by precipitation at the given p_H.

It will be observed that the degree of removal of activity and the weight of precipitate both increase as the p_H rises above 1. At the same time these two factors are not closely parallel, since 88 % of the enzyme has been removed at p_H 2·75 although the weight of precipitate is only 24 % of that found at p_H 4·85. Indeed, the actual separation of insoluble matter is spread over a range of about four p_H units while the precipitation of activity is a phenomenon localised fairly definitely about p_H 2 (Fig. 3). This is of interest in view of the fact that a number of observers have reported a critical point for pepsin—isoelectric point, point of optimum adsorption, etc.—within the region of p_H 2·5 [Michaelis and Davidsohn, 1910; Northrop, 1920; Kikawa, 1926; Forbes, 1927; Fenger and Andrew, 1927]. The curve reproduced in Fig. 3 is that of a hypothetical hydrion dissociation of pepsin of pK 1·9, and

the alignment with the observed degree of precipitation of the activity may be taken to suggest that this reaction of pepsin with safranine is conditioned by the acid-base relations of the reactants. It is of course quite probable that this pK is a characteristic of the inert material with which the enzyme is

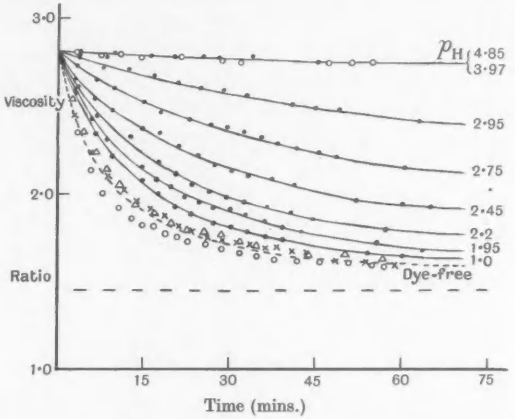

Fig. 2. Effect of p_H on precipitation of gastric juice by safranine (activity of supernatant liquors). $[D]=0{\cdot}2\,\%$. $[E]=0{\cdot}175\,\%$.

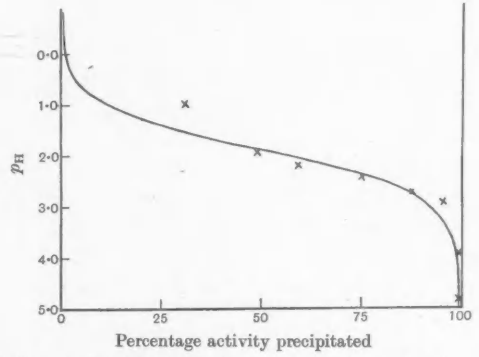

Fig. 3. Relation of precipitated activity to p_H (safranine and dog's gastric juice). The curve is that for a hypothetical hydrion dissociation of pK 1·9.

closely associated rather than of the enzyme itself. The divergence from the curve at p_H 1 may plausibly be attributed to the effect of the weak second basic group of the dye modifying the active mass of safranine at this p_H. In any case, "percentage activity" is a reciprocal term permitting considerable error below values of 50 %.

Purification of pepsin.

The following observations are of interest.

(1) An 8 % solution of a dry preparation of pepsin (Harrington and Co.) after dialysis against running water retained 95 % of its activity and lost 62 % of its solids. The neutral red precipitate from the dialysed solution contained the whole of the activity in 3 % (inclusive of dye) of the original dry weight.

(2) Neutral red precipitates from gastric juice containing the whole of the original activity weighed, according to the p_H of precipitation, from 25 to 90 % (p_H 3 to 4·8) of the dry weight of the original preparation.

(3) The minimum efficient concentration of dye was found to be 0·1 %, and the optimum p_H for purification just above 3.

(4) The weight of inactive matter precipitated from Benger's trypsin by safranine was 17 % of the total.

Summary.

1. The observation of Marston and others is confirmed that the addition of safranine or neutral red to a solution of pepsin or trypsin leads to the separation of a flocculent precipitate. In the case of pepsin solutions, including gastric juice itself, the precipitate removed the peptic activity from solution. The supernatant liquors from the trypsin precipitates, on the other hand, lost no significant proportion of their activity.

2. The optimum conditions of concentration and p_H for the complete removal of pepsin from solution by this means have been determined and a method is described for the recovery of the precipitated pepsin.

3. It has been shown that azine dyes do not "poison" the catalytic activity of either pepsin or trypsin. Consequently there exists in solution under the experimental conditions no significant concentration of a specific protease-azine complex antagonistic to protein hydrolysis.

4. These results give no support to the view of Marston that the azine nucleus reacts specifically with a protease with the formation of a protease-azine complex. The formation of precipitates when azine dyes are added to protease preparations may not be used as an argument either for the piperazine structure of proteins or for any structural scheme of the mechanism of protease digestion.

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LXXII. NOTE ON THE ABSORPTION SPECTRUM OF VITAMIN A.

By OTTO ROSENHEIM and THOMAS ARTHUR WEBSTER.

From the National Institute for Medical Research, Hampstead, N.W. 3.

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It has been claimed repeatedly that vitamin A is characterised by a selective absorption in the ultra-violet region at about 320–328 $\mu\mu$ [Takahashi *et al.* 1925; Morton and Heilbron, 1928]. We have subjected this claim to the experimental test by preparing dehydroergosterol, a sterol with four double bonds, from ergosterol [Windaus and Linsert, 1928]. Spectroscopic measurements by a photographic method [Bourdillon, Fischmann, Jenkins and Webster, 1929], kindly carried out by Mr R. G. C. Jenkins, showed that this substance possessed an intense absorption exactly in the same region as that claimed for vitamin A. The absorption curve agrees with that previously obtained by a photo-electric method [Windaus and Linsert, 1928].

The free sterol as well as its acetate, however, proved to be devoid of growth-promoting properties. Incidentally, we prepared the peroxide by the action of white light on dehydroergosterol in the presence of eosin [Windaus and Linsert, 1928] and found it equally inactive. For the biological tests the substances were administered in olive oil to rats on a vitamin A-free diet in doses from 0·001 mg. to 1 mg. *per diem*, vitamin D being supplied as irradiated ergosterol.

The fact that these substances also fail to give a blue colour with $AsCl_3$ (or $SbCl_3$) is significant in view of the assumed association of this colour reaction with vitamin A. It would seem therefore that selective ultra-violet absorption at 320–328 $\mu\mu$, at any rate by itself, cannot be taken as a criterion of vitamin A.

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LXXIII. THE NATURE OF THE VITAMIN A CONSTITUENT OF GREEN LEAVES.

By DOROTHY LOUISA COLLISON, ELEANOR MARGARET HUME, IDA SMEDLEY-MACLEAN and HANNAH HENDERSON SMITH.

From the Departments of Experimental Pathology and Biochemistry, The Lister Institute, London.

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In spite of the fact that green leaves form one of the most potent sources of vitamin A, comparatively little work has been done on the isolation of the active substance from these leaves. The material from an ethereal extract of spinach or other green leaves has been added to a synthetic diet in order to supply vitamin A by various observers, amongst them Osborne and Mendel [1919, 1, 2] and Willimott and Wokes [1927]. In some experiments which were being carried out by E. M. Hume and H. H. Smith, the unsaponifiable matter from which the sterol had been separated was used to supply the vitamin A constituent of the diet, and it seemed desirable to obtain as much knowledge as possible about the nature of the extract used.

Relation of greenness to vitamin A.

The work of Osborne and Mendel [1919, 1, 2] and Steenbock and Boutwell [1919] had indicated an association of the fat-soluble vitamin with the green parts of plants. This view was examined by Coward and Drummond [1921], who reported that vitamin A was not synthesised by etiolated shoots but that green leaves were active in its formation. Wilson [1922], on the other hand, found that etiolated shoots if given in sufficient quantity could supply the fat-soluble vitamin and that this factor was therefore formed in the absence of light. Coward [1923] concluded that, although the presence of chlorophyll was unnecessary, the formation of the fat-soluble vitamins required the action of light though not of ultra-violet radiation, and later [1925] made the interesting observation that green leaves which in the autumn had turned yellow were more active than the original green leaves. Wilson's work has been confirmed by Coward [1927, 1, 2], Moore [1927] and Heller [1928] and there seems now to be conclusive evidence that, although the green shoots show far more vitamin A activity than the etiolated ones, the latter can supply the active factor if they are given in sufficient quantity. The potency of the shoots is much increased under the influence of light and it cannot be considered as quite certain whether the activity of the etiolated shoots is

due to some effect of germination or to the experimental difficulty of ensuring that no light whatever shall reach the seedlings. A similar difference has been described between the green and white leaves of cabbage. Coward and Drummond [1921] noted that green cabbage leaves were more potent than white leaves in promoting the growth of rats on a diet deficient in the fat-soluble vitamins and Hume [1921] made similar observations for guinea-pigs. Steenbock and Sell [1922] reported that the green inside leaves of cabbages which had failed to head properly contained ten times as much pigment as white leaves on the inside of good heads and were far richer in what was then known as vitamin A, but at the same time they thought that more than the minimum demonstrable amounts were present in white cabbage.

Since the very active green leaves, and the inactive or very feebly active white leaves of cabbage may be obtained at the same time from the same plants, the unsaponifiable matter obtained from the lipoid extracted from white and green leaves respectively seemed very interesting material for investigation and we set out therefore to prepare unsaponifiable matter from both sets of leaves, to examine its chemical nature and to test it biologically for vitamin A activity.

In the present investigation a preliminary study of the white and green leaves of the large cattle cabbage led to the conclusion that three to four times as much unsaponifiable matter could be extracted from a given weight of green as from an equal weight of white cabbage. Not only was more lipoid matter extracted by light petroleum from the dried leaves, but the proportion of unsaponifiable matter derived from it was higher. It was just possible that the results of previous workers might be explained by the fact that equal weights of green and white cabbage contained very different amounts of unsaponifiable matter, although the nature of the unsaponifiable matter was identical.

The method of biological experiment.

In an investigation such as the present one, it is clear that the ultimate criterion for the vitamin A value of the materials studied must be the biological test, although many writers have found the latter far from satisfactory. In carrying out the series of tests described which has extended over about a year, a preliminary depletion period was always used and the aim at first was to obtain from the rats, on various doses of the materials to be tested, growth performances which should be comparable. As time went on, however, information accumulated as to the exact size of the minimal dose on which life could be sustained. It was then found that a better comparison could be made by ascertaining the dose which could just sustain life over a measured period and that which failed to do so. In this way it was possible to say, for instance, that the effective dose of one sample of carotene lay between 0·005 and 0·001 mg., while that of another lay between 0·01 and 0·005 mg. In working with doses so far sub-optimal the difficulty is encountered that

the number of animals which fail to recover when dosage begins after the depletion period is increased; for, when the animal is in a precarious state, the dose needed for recovery is often in excess of that just needed for maintenance. Also there is frequently a latent period of several days before the dose begins to act, in the course of which the animal may become very much worse and it is impossible then to tell whether the dose should be increased or not. The dose may be increased without there being a real necessity to do so or failure to increase it may bring about the loss of a specially prepared animal.

In the last series of experiments (spinach-carotene group) about to be described the results were very much impaired by the frequent occurrence in the animals of haematuria and of septic glands in the neck. Several fulminating cases of corneal ulcer also occurred about this time: it might be that the use of minimal doses favoured the development of the conditions; such was found to be the case independently by McCarrison [1927] and Fujimaki [1926], particularly in favouring the development of stone; but it seems more likely that, since in some cases the condition developed before dosage had begun, *i.e.* somewhere about the 7th week of deficient diet, it was also due to the implantation upon the deficient animals of an infection not previously observed among the experimental animals in this laboratory although frequently described by other workers [Macy *et al.*, 1927].

Where possible an attempt was always made to cure a rat which failed during the experimental treatment, by giving cod-liver oil or other material rich in vitamin A; when the attempt succeeded a positive control was thus supplied but when it did not succeed the failure was not significant.

The diet with addition of a source of vitamin B complex was as follows:

Heated caseinogen	300 parts
Wheat starch	750 ,,
Irradiated hardened cotton-seed oil ...	225 ,,
Salt mixture [Hume and Smith, 1928] ...	75 ,,
Lemon juice	75 ,,
Distilled water	900 ,,

From 10 to 25 g. per head per day were eaten, 14 g. being a usual amount. The fractions to be tested for vitamin A were made up in the cotton-seed oil and administered separately with a pipette.

In previous experiments in which estimations of vitamin A have been made by Hume and Smith [1928], marmite has been relied upon as the source of the vitamin B complex, used in such an amount that an average daily consumption for a rat would be about 0·5 g. About the time when the present series of experiments was begun (July 1928) a number of cases was encountered where rats failed to respond by growth to doses of vitamin A believed to be adequate. These cases remained puzzling until one rat also developed skin lesions closely resembling those found in rats on a diet deficient in vitamin B_2 [Goldberger and Lillie, 1926; Chick and Roscoe, 1927]. Dried yeast auto-

claved at 120° for 5 hours, in which the antineuritic factor is almost completely destroyed while vitamin B_2 survives [Chick and Roscoe, 1927], was kindly supplied by Dr Chick and administered in amounts of 0·5 g. daily. Growth was restored and the dermatitis healed; the conclusion was drawn that marmite could not any longer be relied on as a constant source of supply of vitamin B_2. In the present series of experiments, marmite (75 parts in the above diet mixture) was at first retained and supplemented with 0·5 g. of the autoclaved dried yeast to every 10 g. of the wet diet. The animals used in the experiments, the results of which are given in Table I, received the vitamin B in this way. The animals used in the experiments, the results of which are given in subsequent tables, received no marmite in their diet. In those used in Table II 6 % of dried yeast from the Pharmaco Chemical Products Co. Ltd. was added to the wet diet, and in the final experiments on carotene this amount was increased to 10 or 12 %. The higher amounts appeared to give a more satisfactory result and also made it possible to rely more confidently upon the condition of the eyes as an index of vitamin A deficiency.

Preparation of lipoidal material from cabbage.

The method of preparing the material has already been described [Clenshaw and Smedley-MacLean, 1929]. The preliminary dipping of the leaves in boiling water, which is there described as rendering the leaves more brittle and more readily pulverisable, was used in preparing most of the material used for this investigation. It has however the disadvantage of setting free the chlorophyll so that this is also extracted in the light petroleum solution. If the leaves are dried at the ordinary temperature without the preliminary dipping in hot water, the light petroleum extract is yellow and is practically free from chlorophyll [Arnaud, 1885]. We have since found that a preliminary drying *in vacuo* before drying in the hot room also liberates the chlorophyll, but if the leaves are air-dried at a temperature of 37° the petroleum extract is yellow and appears to be free from chlorophyll. In nearly all the material used in this investigation, the leaves were subjected to the preliminary dipping in hot water, the petroleum solutions were deep green in colour and the lipoidal material contained chlorophyll. Treated in this way, 715 g. of dried white cabbage leaves yielded 7·58 g. of light petroleum-soluble material, whilst from 1300 g. of dried green cabbage leaves 25·83 g. of lipoidal material were extracted.

The material obtained from the green leaves was first tested for its activity in vitamin A. The medium chosen to dissolve the given extract was the same hardened cotton-seed oil as was used in the basal diet. In this preliminary experiment one set of doses was also given in paraffin oil in order to see if these were equally well absorbed. On the whole the effect seemed rather better with the hardened cotton-seed oil, especially in the case of the smaller doses and this medium was used in all later experiments. The results

are shown in Table I, which also contains the record of an experiment in which some dried green spinach leaves which had been several times extracted with light petroleum were extracted with acetone. Since xanthophyll is soluble in acetone and insoluble in light petroleum, this extract would contain the xanthophyll present in the leaves. No evidence of any vitamin A activity was found in this fraction, a result in agreement with that observed by Willimott and Moore [1927].

Table I. *Test of green cabbage and acetone-spinach extracts for vitamin A activity.*

Material tested	Dose mg.	No. of litter	No. of rat	Sex	No. of days maintenance	Growth in period of experiment g.	Notes
Green cabbage extract	5·0	1044	516	♀	28+	43	—
	„	1045	518	♀	28+	43	—
(*a*) In cotton-seed oil	„	„	519	♂	28+	66	—
	2·5	1044	517	♀	25+	15	Following larger dose for 15 days
	„	1045	525	♀	25+	15	Following larger dose for 14 days
	0·5	1044	515	♀	21+	25	Following larger dose for 4 days
	„	1045	523	♀	21+	13	Following larger dose for 5 days
(*b*) In paraffin oil	5·0	1044	512	♀	28+	43	—
	„	1045	524	♀	28+	40	—
	„	„	520	♂	28+	61	—
	2·5	1044	510	♀	18	3	Following acetone-spinach extract, died respiratory disease
	„	„	514	♀	25+	10	Following larger dose for 11 days
	0·5	1045	526	♀	21+	9	Following larger dose for 5 days
Acetone-spinach extract	10·0	1044	510	♀	8	2	Rapidly deteriorating, cured by green cabbage extract
						−8	
	„	1045	527	♀	14	2	Deteriorating, cured by other material
						−5	
	„	„	521	♂	15	−7	Deteriorating, cured by other material

Note. Behaviour of rats when the material tested for vitamin A is supplied after a preliminary depletion period of 5–6 weeks. The growth or maintenance is for a measured period of 28 days though in one or two cases the period was arbitrarily shortened from lack of material or other cause. Where the symbol + fails to follow the days of maintenance it is indicated that a decline set in. Success or failure to maintain should be taken as the criterion and the amount of growth taken as a subsidiary comment.

Preparation of the unsaponifiable material.

This was prepared from the light petroleum-soluble material by adding to its ethereal solution at laboratory temperature an excess over the calculated amount of an alcoholic solution of sodium ethoxide, and allowing it to stand overnight. On diluting and extracting the solution with ether the chlorophyll remains in the aqueous alkaline solution.

From the green cabbage, 10·9 g. of unsaponifiable matter were obtained, a yield of 0·84 % of the dried leaves; the iodine value (Hübl) was 110·2. The white cabbage gave 1·78 g. of unsaponifiable matter, a yield of 0·25 % of the dried leaves; its iodine value was 60·5. The green leaves gave therefore three to four times as much unsaponifiable matter as an equal weight of white cabbage and the substance obtained from the green leaves was much more unsaturated than that prepared from the white cabbage.

Quantities of 1 g. of the unsaponifiable material obtained respectively from the green and white leaves were dissolved in a small quantity of ether and added to 25 g. of hardened cotton-seed oil. The ether was then blown off from the melted fat by a current of CO_2.

The results of the biological tests are given in Table II.

Table II. *Test of unsaponifiable matter from green and white cabbage for vitamin A activity.*

See note under Table I.

Material tested	Dose mg.	No. of litter	No. of rat	Sex	No. of days maintenance	Growth in period of experiment g.	Notes
Unsap. matter from green cabbage	1·5	1142	531	♂	28+	49	—
	1·0	,,	532	♀	28+	31	—
	,,	,,	535	♀	28+	21	—
	,,	1160	548	♂♀	—	—	Died at once
	0·5	1142	533	♀	28+	22	Survived a pregnancy, litter lost
	,,	1160	543	♀	28+	41	—
	,,	,,	547	♂	28+	48	—
	0·25	,,	538	♀	28+	23	Survived a pregnancy, litter lost
	,,	,,	541	♀	28+	26	—
	,,	,,	544	♀	28+	28	—
Unsap. matter from white cabbage	10·0	1160	539	♀	28+	16	—
	5·0	,,	542	♀	11	−14	Cured by green unsap.
	3·0	,,	540	♀	24	21 / −10	Maintained to 28th day by increasing dose to 10 mg.
	,,	,,	545	♂	—	—	Died at once
	,,	,,	546	♂	14	15 / −22	Died
	1·5	1142	530	♂	16	9 / −27	Moribund, killed
	1·0	,,	534	♀	28+	13	—
	,,	,,	536	♀	15	−25	Very ill, cured by green unsap.
	0·5	,,	537	♀	14	−34	Pregnant, died
Green cabbage unsap. Fraction insol. in hot and cold alcohol. Sol. in light petroleum	1·2	1162	553	♂	28+	56	—
	0·6	,,	555	♂♀	28+	35	—
	0·2	,,	550	♀	28+	15	—
	,,	,,	551	♀	28+	23	—
	0·03	,,	554	♂	23+	8	—

Fractionation of the unsaponifiable material.

Having established that the green leaves yielded an unsaponifiable matter at least ten times as potent in vitamin A activity as the white, the next step

was to fractionate this material and to find out how far this activity could be concentrated. The first method used was by extraction with different solvents.

Cold alcoholic extract. The unsaponifiable fraction was twice extracted with cold alcohol and, from the cold alcoholic extract of 5 g. of the original unsaponifiable matter, crystals of a substance melting at 135° separated on standing; these gave a positive Salkowski reaction and probably consisted of a sterol. A very small amount of red crystals was also separated from this extract; the filtrate from these was evaporated to dryness and yielded 2·3 g. of residue having an iodine value of 130·7.

Hot alcoholic extract. The substance insoluble in cold alcohol was three times extracted with hot 96 % alcohol. From this crystals separated melting from 63° to 68°. By subsequent recrystallisation from alcohol white crystals melting at 75° were obtained, and a substance melting up to 68°. A preliminary investigation of this fraction was described by Clenshaw and Smedley-MacLean [1929], who isolated a hydrocarbon which they regarded as identical with the hentriacontane, $C_{31}H_{64}$, M.P. 68°, which they had already isolated from spinach. Subsequently Channon and Chibnall [1929] published the results of an investigation on cabbage and described the isolation of a hydrocarbon melting at 62·7°–62·8° and of a ketone $C_{29}H_{58}O$ melting at 80·5–81°; these they identified respectively as nonacosane and di-*n*-tetradecyl ketone. The hydrocarbon isolated by Clenshaw and Smedley-MacLean from spinach melted sharply at 68–68·5° and was analysed and satisfactorily identified as the hydrocarbon $C_{31}H_{64}$. The substance which they obtained from cabbage, melting at 68°, was only separated in very small amount: it was not analysed, and for its identification a mixed melting-point with the hydrocarbon obtained from spinach was relied upon. In the present investigation, working with a green Christmas cabbage, we isolated two substances melting at 63° and 80° to 81° respectively and confirm the results published by Channon and Chibnall. We propose to examine again the large white summer cattle cabbage used in their investigation by Clenshaw and Smedley-MacLean in order to determine definitely whether the substance isolated by them was identical with the hentriacontane of spinach or was a mixture of the two substances identified by Channon and Chibnall.

Light petroleum extract. A small amount of blackish oily residue (iodine value, 173) remained which was practically insoluble in hot and cold alcohol: this was extracted with warm light petroleum (B.P. 40° to 60°) and from the solution red crystals of carotene separated. After several recrystallisations from light petroleum 0·018 g. crystals, which softened at 174° and melted at 178°, were obtained; they had an iodine value of 324. Very much less of the material from the white leaves was available than from the green: the whole of it was found to be soluble in alcohol and there was no blackish residue, corresponding to that obtained in working up green cabbage, insoluble in alcohol and soluble in light petroleum and rich in carotene. The quantity of

carotene present must have been so small as to have been completely dissolved in the alcoholic solutions.

Colour test with antimony trichloride. These various fractions were tested by the method of Carr and Price [1926] with the following results.

Green cabbage:

(1) Unsaponifiable matter	Blue-green colour
(2) Cold alcohol extract, 0·016 g. per cc.	Blue-green colour
(3) White substance from hot alcohol extract, 0·020 g. per cc.	Colourless
(4) Ether extract, insoluble in alcohol, 0·003 g. per cc.	Green colour
(5) Carotene crystals, 0·001 g. per cc. ...	Permanent blue colour
(6) Carotene crystals, 0·0001 g. per cc.	Permanent blue colour

White cabbage:

(7) Unsaponifiable matter	Blue-green colour, much less intense than (1)

The fact that solutions of carotene give a blue and not a green colour may be interpreted as showing that the yellow colour of carotene disappears and that it is therefore the yellow coloured carotene itself that enters into combination with the antimony trichloride solution. The green colour produced by the yellow unsaponifiable fractions which contain carotene must therefore be ascribed to a mixture of the blue compound formed from carotene and antimony trichloride with a yellow substance which remains yellow and is not changed by the antimony trichloride solution.

Preparation of carotene.

It appeared from the above results that the activity was associated with the carotene-containing fractions, and was certainly greatest in the most unsaturated fractions. It seemed desirable therefore to isolate carotene from the unsaponifiable matter and to determine whether the vitamin A active fraction was still associated with the carotene crystals. Three materials were selected, cabbage, spinach and carrots, and from each of these a small specimen of carotene was prepared.

From cabbage. 10·9 g. of the unsaponifiable matter from green cabbage were fractionated as described above. A very small amount of carotene was obtained from the fraction soluble in hot alcohol: the fraction insoluble in alcohol but soluble in ether deposited dark reddish crystals which were several times recrystallised and finally obtained softening at 174° and being completely melted at 178°. Examination of a dilute solution of this carotene in light petroleum solution showed the presence of two bands extending from 450 to 455 $\mu\mu$ and from 478 to 486 $\mu\mu$ respectively.

From spinach. When similarly treated 11·05 g. of the lipoidal matter from spinach yielded 5·09 g. of unsaponifiable matter (I.V. 108). In this case the carotene was separated by dissolving the unsaponifiable fraction in carbon disulphide and fractionally precipitating it with alcohol. The earlier precipitates contained most of the impurities; the later (I.V. 183) were rich in carotene and were redissolved in carbon disulphide, again fractionally precipitated with alcohol, and then several times recrystallised from ether. The iodine value was 352. After further recrystallisation, the material melted at 163–164° and had an iodine value of 338. This fall in value cannot be regarded as significant since very small quantities of material were used for the determinations.

Part of the material (I.V. 183) was recrystallised from ether, dissolved in light petroleum, shaken up with chalk and filtered through a layer of chalk. The solvent was then evaporated from the filtrate and the residue used for a biological test on one rat.

From carrots. Slices of carrots were dried at 37° and extracted with light petroleum. The residue from the petroleum extract (I.V. 142·3) was worked up similarly to the unsaponifiable matter from spinach. The reddish yellow crystals first obtained had an iodine value of 181°. By fractionally precipitating with alcohol from their carbon disulphide solution, crystals were obtained melting from 164° to 170° (I.V. 220). By further purification 22 mg. recrystallised carotene of iodine value 300 were obtained. Examined microscopically it consisted of characteristic red diamond-shaped crystals; at this stage it was used for the biological tests, and was compared with the cruder specimen of iodine value, 220.

Solutions (0·14 %) of all three specimens of carotene in hardened cottonseed oil gave a blue colour with the antimony trichloride reagent. A 0·07 % solution of the specimen from cabbage was also tested and gave a marked blue colour.

The evidence at present available as to the constitution of carotene, $C_{40}H_{56}$, indicates that it possesses 11 double bonds of which probably 8 are conjugated [Zechmeister and v. Cholnoky, 1928]. The iodine values for the specimens of carotene now examined varied between 300 and 350, corresponding to about 7 ethylenic linkages in the molecule. It seems unlikely that 22 halogen atoms would be added by interaction with the iodine reagent. It is known for instance that ethylenic linkages in the α–β position to an OH or COOH group do not react with the iodine reagent [Smedley-MacLean and Thomas, 1921], and the addition of so many halogen atoms would probably modify the reaction. The variations from 300 to 352 cannot be regarded as significant in view of the very small quantities of material which were used for these determinations; the experimental error was large, since only 2–3 mg. were available for the test, and might well be 10 % of the observed value.

The highest melting point of the various specimens of carotene was that from cabbage which softened at 174° and finally melted at 178°.

Table III. *Test of carotene for vitamin A activity.*

See note under Table I.

Material tested	Dose mg.	No. of litter	No. of rat	Sex	No. of days maintenance	Growth during period of experiment g.	Notes
Cabbage carotene:							
I.V. 330	0·01	1228	572	♀	28+	40	— —
M.P. 174–178°	,,	1227	577	♂♀	28+	76	— —
	0·005	,,	579	♀	28	7	Moribund at end of experiment with septic bladder condition
	,,	,,	578	♂	28+	60	— —
	0·003	1223	563	♂♀	28+	25	— —
	,,	1225	601	♂	28+	10	Following smaller dose, septic glands in neck
	0·002	1223	564	♂	28+	16	— —
	,,	1275	601	♂	6	− 5	Following smaller dose, changed to larger dose
	0·001	1223	561	♀	24	6 −15	Maintained to end of period on 0·002 mg.
	,,	1228	574	♂♀	4	− 2	Cured by cod-liver oil
	,,	1275	604	♂♀	3	− 5	Died—septic uterus
	,,	,,	605	♀	14	5 − 4	Haematuria
	,,	,,	601	♂	6	− 5	Changed to larger dose
Carrot carotene:							
(*a*) I.V. 220	0·04	1272	596	♀	28+	29	— —
M.P. 169°	0·02	1223	567	♂	28+	7	Following dose mother-liquor
	,,	1272	600	♀	28+	23	— —
	,,	,,	597	♂	28+	29	— —
(*b*) Recryst.	0·01	1223	560	♀	28+	31	— —
I.V. 300	,,	1228	573	♂	7	− 6	Fulminating corneal sepsis. Treated perhaps prematurely with other material—cured
	,,	1227	576	♂	28+	36	— —
	0·005	1275	607	♂♀	10	− 4	Cured by cod-liver oil
	,,	,,	602	♂	16	—	Changed to 0·01 mg. with temporary improvement
	0·004	,,	608	♀	13	− 8	Cured by cod-liver oil
	0·002	1275	603	♂♀	12	− 8	Cured by cod-liver oil
	0·001	1228	575	♂♀	5	− 4	Cured by other material
	,,	1275	608	♂♀	13	− 8	Changed to 0·004 mg.
	,,	,,	607	♂♀	12	−10	Changed to 0·005 mg.
	,,	,,	602	♂	7	− 8	Changed to 0·005 mg.
Spinach carotene:							
(*a*) I.V. 338	0·012	1300	611	♂	8	—	Haematuria, died, severe stone
M.P. 164°	,,	,,	616	♂	28+	36	Foetid urine
	0·008	,,	609	♀	28+	15	Haematuria before beginning dosage
	,,	,,	610	♀	18	4 −13	Haematuria—died
(*b*) I.V. 352	0·010	,,	613	♂	28+	51	Foetid urine — enlarged gland in neck
	,,	,,	614	♂	8	−10	Haematuria before beginning dosage—died
	,,	,,	615	♂	25	25 −14	Haematuria—recovered with cod-liver oil
(*c*) After shaking solution with, and filtering through, chalk	0·006	1300	612	♂	28+	27	— —

The separation of carotene from cabbage is certainly very incomplete, especially when, as in our separation, no stringent precautions were taken to guard against oxidation. The actual amount of crystals separated must represent only a small proportion of the carotene present in the original cabbage.

Figures are not available as to the minimum weight of fresh or dried cabbage which will maintain a rat on a vitamin-A deficient diet in a good state of health under the conditions of the experiment.

From the figures now given 11·17 kg. cabbage leaf gave 1300 g. dry material from which 25 g. lipoidal substance and 10·9 g. unsaponifiable matter were extracted. In our experiments 0·25 mg. of unsaponifiable matter was more than sufficient for the minimum dose to supply the vitamin A factor and must therefore contain the minimum dose of carotene, here determined as 0·003 to 0·005 mg. This would correspond to rather more than 2 % of carotene in the unsaponifiable matter. Channon and Chibnall [1929] estimate the amount of carotene in the acetone-ethereal extract of cabbage with which they worked as 0·86 % and as the unsaponifiable fraction would be only a portion of their total extract, these figures seem to be of a comparable order.

The biological results are given in Table III.

The specimen of carotene which melted at the highest temperature and was therefore presumably the most pure was that from cabbage which was active in a dose of 0·003 mg. *per diem*: in this group the division between the active doses which maintained life for 28 days with fair growth and those of 0·001 mg. which failed to do so was well brought out. The specimens of carotene from spinach and from carrots respectively were less pure, the melting point was lower and the positive dose required was appreciably higher, being in the neighbourhood of 0·01 mg. The animals were not in such good condition in the later groups used for these experiments and it is not therefore permissible to make the deduction that the purity of the carotene specimen is in inverse ratio to the dose required, but we can say that we found no indication that the activity of the dose diminished with increasing purity of the carotene. The residue from the mother-liquor from which we had separated the carotene crystals from cabbage was tested on three rats and gave negative results with doses of 0·005 and 0·02 mg., doses which were active in the case of the carotene crystals, so that there was a definite concentration of activity associated with the carotene crystals. The residue from which the carotene had been extracted with ether was fed to 5 rats and was inactive in doses up to 0·1 mg.

The material obtained by shaking up an ethereal solution of the spinach carotene in light petroleum with finely precipitated chalk and filtering through a layer of chalk, and evaporating the solvent, showed no diminution in activity; a dose of 0·006 mg. gave a good result but only one rat was available for this test (Table III, no. 612). It seems that the active substance, like the carotene, is not absorbed by chalk.

Discussion.

The results obtained with carotene are closely in agreement with those recently published by v. Euler *et al.* [1928, 1929] and by Moore [1929]. The association of vitamin A activity with the carotenoid pigments has been frequently referred to by different workers. The idea has been supported in a series of papers by Steenbock and Sell [1922], who however do not definitely identify the active factor as carotene: the association of the carotenoids with the active vitamin A substance was discussed by Rosenheim and Drummond [1920] but the identification of the vitamin A as carotene was definitely rejected by them and by Drummond, Channon and Coward [1925]. The latter observers isolated from carrots a specimen of carotene which after being recrystallised four times melted at 167·5°, and which they regarded as free from vitamin A activity, although the dosage given is not stated. More recently Dulière, Morton and Drummond [1929] have separated from carrots a specimen of carotene melting at 184·9° after recrystallising it several times from hexane and working under anaerobic conditions. This preparation was found to be almost inactive even in doses of 0·5 mg.

The fact that both specimens of carotene isolated by Drummond and his colleagues, melting respectively at 167° and 183·9°, were found to be inactive suggests that the inactivity of the purer specimen described in the more recent work is not due to the elimination of an active impurity although the possibility that Dulière, Morton and Drummond have isolated an inactive constituent from the crude carotene cannot be entirely excluded.

All the workers who find that carotene shows vitamin activity include fat in their basal diet, whereas Drummond and his co-workers give a fat-free diet. It is possible that in the latter case the pigment is not so well absorbed and it is interesting to note that Hart, Steenbock, Kletzien and Scott [1927] found that the antirachitic unsaponifiable constituent of cod-liver oil did not establish the calcium balance in a goat unless it was given in solution in a liquid fat such as corn oil, although the corn oil itself had no effect on assimilation[1]. On the other hand, Hume and Smith (unpublished experiment) have found that the unsaponifiable fraction from a light petroleum extract of spinach, freed from sterols, is an adequate source of vitamin A, when dissolved in liquid paraffin and added to a fat-free diet. The latter observation suggests that it may be some element of the unsaponifiable fraction of the fat in the diet, rather than the fat as fat, which is needed in conjunction with carotene, to produce the biological effect of vitamin A.

Evidence appears to be accumulating tending to show that more than one substance can function as vitamin A. Differences in the stability of the vitamin A derived from plant and animal sources have been described [Sherman, Quinn, Day and Miller, 1928], and it seems difficult to accept as

[1] Since writing the above, attention has been drawn to the influence of fat in the diet by Burr and Burr [1929] and McAmis, Anderson and Mendel [1929].

identical the highly coloured crystals which are associated with the vitamin A activity in plants with the much paler substances derived from animal oils. The recent publication of v. Euler, v. Euler and Karrer [1929], which records the inactivity of α-crocetin and the activity of its dihydro-derivative, furnishes evidence in support of the view that the property of vitamin A activity is to be regarded rather as belonging to a special grouping of atoms which may be common to several individual molecules than to a definite molecule.

Dulière, Morton and Drummond have laid stress on the appearance of an absorption band 608–612 $\mu\mu$ in the reaction product of antimony trichloride and specimens of the unsaponifiable matter from cod-liver oil which are very active in vitamin A and have drawn the inference that this band is associated with the actual vitamin A. The absorption spectrum of the blue compound produced when carotene reacts with the same reagent does not show this particular band [v. Euler *et al.*, 1928], though it is suggested that the difference may be due to the presence or absence of an oily medium. If the same vitamin A which produces this band be indeed a contamination in the carotene crystals, as Dulière *et al.* suggest, it is difficult to see why the characteristic 608–612 $\mu\mu$ band does not appear in the absorption spectrum of the carotene compound. Since the carotene crystals are as potent in vitamin A activity as any unsaponifiable fraction from animal oils yet investigated, one would expect the 608–612 $\mu\mu$ band to be as strongly marked in the spectrum of the carotene antimony trichloride compound. If the vitamin A of animal oils be indeed responsible for the production of the band, its absence when the very active carotene crystals are used would furnish additional evidence that the active substance in the cod-liver oil is not identical with that in the active carotene crystals.

Summary.

(1) The vitamin A activity of the unsaponifiable fraction from white cabbage is very small compared with that of the corresponding fraction derived from green leaves; this fraction, however, is active if given in sufficient amount. The respective minimal doses are about 10 and 0·25 mg.

(2) The vitamin A substance of green spinach and cabbage leaves and of carrots is contained in the most highly unsaturated fraction of the unsaponifiable matter and, as far as the process of purification here employed extends, remains associated with the carotene crystals.

(3) The carotene crystals obtained from cabbage softened at 174° and melted at 178°, those from spinach at 163–4° and from carrots at 164–9°. The vitamin A activity is certainly not diminished in the specimen of higher melting point and of therefore presumably greater purity: in this specimen the active dose lies between 0·002 and 0·005 mg. No claim to a great degree of purity in the specimens of carotene separated is made, since the work carried out was on too small a scale to admit of sufficient recrystallisations to ensure the separation of all impurities.

(4) It is possible that the crystals of carotene may themselves be homogeneous and active or that they may consist of two or more closely related substances only one of which possesses vitamin A activity.

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LXXIV. THE DETERMINATION OF CHLORIDE IN ANIMAL TISSUES.

By ERNEST HAROLD CALLOW.

From the Food Investigation Board of the Department of Scientific and Industrial Research and the Low Temperature Research Station, Cambridge.

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During the course of an investigation of salt-cured meat, it became necessary to find a highly accurate method for the estimation of chloride in both salt-cured and fresh meat. It was essential that the method should be applicable to samples as large as 50 g., and that it should be simple and reliable. In order to determine the accuracy of various methods, estimations were carried out of the chloride content of (*a*) fresh meat, and (*b*) an equal weight of the same sample of fresh meat to which had been added a known amount of sodium chloride. Lean pork was minced three times and well mixed. 50 g. portions were placed in well-stoppered glass bottles with wide necks. The contents of half the bottles were analysed in order to determine the chloride content of the fresh meat. Known amounts of sodium chloride were added to the remaining bottles, which were kept at 0° for several days to allow the salt to react with the tissue. The chloride content of the salted meat was then determined.

There are two stages in the chloride analysis of meat. First, the chloride must be extracted from the tissues with as little protein as possible, and secondly the chloride content of the extract must be determined. For the second stage, the Volhard method was used in this investigation. The first stage, namely extraction, is frequently carried out after the organic matter has been charred by heat. This method gives low values for the chloride content because a certain amount of chloride is invariably lost by volatilisation. The results obtained by this method were lower than those obtained by any of the other methods investigated (see Table I). The incineration method has been modified by Weitzel [1920]. He mixed the substance to be analysed with calcium oxide before incineration. There is no doubt that this is an improvement, but even the modified method in our hands gave low results for the chloride of meat (see Table II).

As incineration was found to be unsatisfactory, an attempt was made to find other ways of obtaining the chloride in solution with minimal amounts of protein. It seemed probable that the most promising method would be to use a boiling aqueous salt solution which would extract the chloride and at

the same time coagulate the proteins. Solutions of sodium sulphate and of iron alum were used but gave results which were either too low or inconsistent. Distilled water alone, however, proved to be an excellent reagent for extraction.

Table I. *Typical results obtained for chloride estimation of meat.*

Various methods of extraction were used and in each case the extract was analysed by Volhard's method. The same sample of pork was used throughout.

Method of extracting chloride from meat	Chloride (expressed as g. NaCl) found in 50 g. of lean pork	Chloride (expressed as g. NaCl) found in 50 g. of lean pork to which had been added 2 g. NaCl	NaCl recovered g.	Percentage recovery
Boiled with distilled water	0·055	2·062	2·007	100·4
Boiled with 20 % sodium sulphate	0·053	2·046	1·993	99·7
Boiled with 20 % sodium sulphate plus iron alum	0·056	2·036	1·980	99·0
Boiled with distilled water plus iron alum	0·046	2·002	1·956	97·8
Incinerated at a dull red heat	0·041	1·982	1·941	97·1

Table II.

The same sample of pork was used throughout. In each case the results are the average of two determinations.

NaCl added to 50 g. of pork	Extracted with boiling distilled water			Incinerated with calcium oxide (Weitzel's method)		
	NaCl found	NaCl recovered	Percentage recovery	NaCl found	NaCl recovered	Percentage recovered
g.	g.	g.		g.	g.	
Nil	0·054	←	—	0·051	—	—
1	1·044	0·990	99·0	1·026	0·975	97·5
2	2·051	1·997	99·9	2·003	1·952	97·6

The following procedure was finally adopted. 50 g. of finely minced meat are placed in a 300 cc. beaker and covered with distilled water. The beaker is placed in a boiling water-bath, and the contents are stirred in order to prevent the formation of large lumps during the coagulation of the protein. The beaker is then heated over a Bunsen burner until the contents boil. The precipitate is allowed to settle and the clear fluid filtered into a flask. The residue is again boiled with distilled water, and the solution decanted and filtered into the collecting flask. In the case of fresh meat this process is repeated 5 or 6 times and the filtrate is transferred to a 1000 cc. standard flask. With salted meat it is necessary to boil the residue 10 or 12 times with distilled water. The filtered solution in this case is made up to 2000 cc. Measured volumes of this solution (with fresh meat and with very low concentrations of sodium chloride the solution must be concentrated by evaporating 250 cc. to about 10 cc.) are then placed in boiling-tubes with an excess of silver nitrate. A volume of concentrated nitric acid equal to the volume of the mixture is added, and the tubes are heated in a boiling water-bath for

an hour and a half to destroy the proteins. When cool, distilled water is added to dilute the strongly acid solution. The silver chloride is filtered off and washed, and the excess of silver nitrate is estimated by the Volhard method.

A typical series of results is given in Table III.

Table III.

The same sample of pork was used throughout.

Weight of NaCl added g.	Total NaCl in 50 g. meat g.	NaCl recovered g.	Percentage recovery
Nil	0·053, 0·051, 0·048 } average 0·051		
0·2	0·246	0·195	97·5
0·2	0·248	0·197	98·5
0·5	0·552	0·501	100·2
0·5	0·553	0·502	100·4
1·0	1·050	0·999	99·9
1·0	1·055	1·004	100·4
2·0	2·051	2·000	100·0
2·0	2·041	1·990	99·5

These results show that sodium chloride which has been added to meat can be estimated quantitatively by this method. Although there is complete recovery of added sodium chloride, it does not necessarily follow that this method accurately estimates the chloride originally present in the meat. In order to test this point, the following experiments were carried out. After extracting the chloride by the method described above, the residues were cautiously incinerated at dull red heat. The charcoal formed was extracted with water and then ashed. No trace of chloride was detected in the charcoal extract or ash. Even when the extracted residues from 300 g. of fresh meat were bulked together for this purpose, no chloride could be detected. This indicates that the chloride is completely extracted from meat by this method. The question then arises as to whether the method is equally reliable when applied to commercially salt-cured meats, which contain saltpetre and often sugar in addition to sodium chloride. Estimations carried out on similar samples of such material gave remarkably concordant results, considering the difficulties of sampling. For example, six estimations of a sample of minced ham gave the following results for the concentration of chloride expressed as sodium chloride: 8·04 %, 8·02 %, 7·96 %, 7·88 %, 8·04 % and 8·02 %. Four estimations of a sample of cooked ham gave the following results: 4·94 %, 4·84 %, 4·84 %, 4·84 %. After extraction and incineration, the coagulated residue showed no trace of chloride.

An alternative method of estimating small quantities of chloride in biologica fluids has been put forward recently by Christy and Robson [1928]. They add $N/29{\cdot}25$ silver nitrate to the solution in the presence of nitric acid; the silver chloride is filtered off, and the excess of silver nitrate in the filtrate is titrated against $N/29{\cdot}25$ potassium iodide in the presence of potassium

bi-iodate and soluble starch. Silver iodide is precipitated and, when the reaction is complete, further addition of potassium iodide results in the formation of iodine. According to Christy and Robson, the slightest excess of potassium iodide results in the formation of the well-known blue colour of starch iodide. I have found, however, that the end-point is obscured by the appearance of a yellow colour which changes the clear blue to a muddy green. Moreover, I found that the end-point is not reached until a considerable excess of KI has been added.

In order to test the method, I carried out preliminary analyses on a solution containing a known amount of sodium chloride. I followed Christy and Robson's procedure except that I used $M/100$ potassium bi-iodate, as it is not clear what is meant by $N/100$ potassium bi-iodate. In every case the end-point of the titration was greenish and not well defined, and the results were 5–10 % too low. A large number of control titrations were then carried out in order to discover the cause of these grave discrepancies. Using 10 cc. of $N/29{\cdot}25$ silver nitrate, no end-point was obtained until more than 11 cc. of $N/29{\cdot}25$ potassium iodide had been added. In Christy and Robson's method 1 cc. of $N/100$ potassium bi-iodate is used, but I found that the

Table IV.

$N/29{\cdot}25$ silver nitrate cc.	$M/100$ potassium bi-iodate cc.	$2\,N$ nitric acid cc.	1 % soluble starch cc.	Water added cc.	Potassium iodide cc.
10	1·00	10	2	90	11·8
10	0·50	10	2	90	10·8
10	0·20	10	2	90	10·45
10	0·10	10	2	90	10·31
10	0·05	10	2	90	10·20
10	1·00*	10	2	90	10·92
10	0·50*	10	2	90	10·63
10	0·20*	10	2	90	10·26
10	0·10*	10	2	90	10·20
10	0·05*	10	2	90	10·30
10	1·0	10	1	90	11·81
10	1·0	5	1	95	11·54
10	1·0	2	1	98	11·26
10	1·0	1	1	99	10·92
10	1·0	0·5	1	99·5	10·80
10	1·0	0·2	1	99·8	10·36
10	1·0	0·1	1	99·9	10·32
10	1·0	10	2	90	12·02
5	1·0	10	2	90	6·47
2	1·0	10	2	90	2·91
1	1·0	10	2	90	1·66
0·5	1·0	10	2	90	0·84
Nil	1·0	10	2	90	0·05
10	1·0	10	1	90	11·72
10	1·0	10	1	80	11·90
10	1·0	10	1	60	11·87
10	1·0	10	1	40	11·78
10	1·0	10	1	20	11·80
10	1·0	10	1	10	11·92
10	1·0	10	1	Nil	11·93
10	0·10	10	1	Nil	10·30

* KIO_3.

results agreed more and more closely with the expected figure as the concentration of bi-iodate was decreased until, in the presence of 0·10 cc., only 10·2 cc. of potassium iodide were needed for 10 cc. of silver nitrate. The end-point became progressively sharper as the concentration of bi-iodate was decreased, the muddy green colour being gradually replaced by a clear blue colour. With less than 0·05 cc. of $M/100$ potassium bi-iodate the end-point was indefinite because the blue colour was too pale. When potassium iodate was used instead of the bi-iodate, similar results were obtained. Other control titrations involved variations in the amounts of silver nitrate, nitric acid and water (see Table IV). The titration values of potassium iodide necessary to give an end-point are given in the last column.

On titrating in the absence of starch and filtering off the silver iodide formed, the filtrates obtained were yellow to orange-brown in colour. It was naturally suspected that the yellow colour was due to the formation of iodine, but this hypothesis at first appeared untenable because of the non-appearance of a blue colour with starch. Further experiments, however, indicated that iodine was actually present. It could be extracted by means of chloroform or obtained as a sublimate in the condenser on vacuum distillation. Since the yellow colour was due to iodine, it may be asked why no blue colour was obtained with starch. The answer to this question is the usually neglected fact that a solution of iodine in water is incapable of giving a blue colour with starch, except in the presence of a soluble iodide [Treadwell, 1921].

Thus, when solid iodine is allowed to remain in contact with a solution containing soluble starch, nitric acid and potassium iodate (or bi-iodate), no blue colour appears. Under these conditions, even if HI is formed, it immediately reacts with the iodate and nitric acid to give free iodine:

$$5HI + KIO_3 + HNO_3 = 3I_2 + 3H_2O + KNO_3.$$

The fluid gradually becomes yellow and then orange-brown, and iodine can be extracted by shaking with chloroform. In all respects this fluid is similar to the yellow or orange-brown fluid previously described as occurring in the control titrations after filtering off the silver iodide. In the control titrations potassium iodide is run into a solution containing silver nitrate, potassium bi-iodate (or iodate), nitric acid and soluble starch. The blue colour of the starch iodide does not appear at the point when all the silver has been precipitated (as silver iodide) because further additions of potassium iodide react with the iodate or bi-iodate and nitric acid to give free iodine. This iodine gives no blue colour with starch because there is no soluble iodide present. From theoretical considerations we should not expect that a blue colour would be formed until enough potassium iodide has been added to decompose all the potassium bi-iodate present in the solution. In actual practice, however, it has been found that when starch is present the blue colour is formed before the theoretical amount of potassium iodide has all been added (see Table IV). This appears to be due to the fact that starch

iodide is a relatively stable compound and when once it has been formed it is difficult to decompose. This point is illustrated by the following experiment. When a large excess of potassium iodate (or bi-iodate) is added to a trace of potassium iodide in a solution of nitric acid, iodine is formed and all the potassium iodide is decomposed. On adding starch, no blue colour is produced. If however starch is added to the potassium iodide and nitric acid before the iodate or bi-iodate, a deep blue colour is immediately produced.

Christy and Robson's method is therefore subject to two sources of error. In the first place a considerable amount of free iodine may be present before the blue end-point is reached, and in the second place the excess of potassium iodide required varies with the concentrations of the reacting substances. It is conceivable that it might be used as an empirical method, but only under rigidly standardised conditions. This fact was emphasised by Bang [1913], who devised the procedure which forms the basis of Christy and Robson's method.

SUMMARY.

(1) A method is described for the complete extraction of chloride from animal tissues.

(2) Chloride in such extracts was estimated with a high degree of accuracy by means of a modification of Volhard's method.

(3) A criticism is given of Christy and Robson's method for the estimation of chloride in biological fluids.

I gladly take this opportunity of thanking Sir William B. Hardy, F.R.S., for the interest he has taken in this work. I also wish to thank Mr David Gatherum for carrying out many of the analyses recorded in this paper.

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LXXV. THE SALT EFFECT ON THE INDUCTION PERIOD IN THE FERMENTATION BY DRIED YEAST.

By HIDEO KATAGIRI and GOHEI YAMAGISHI.

From the Department of Agriculture, Kyoto Imperial University, Japan.

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The effect of a number of substances on the period of induction during which no fermentation is observed with the mixture of sugar solution and zymase preparations has been observed by many authors. Meyerhof [1918] pointed out that with maceration extract this period was completely abolished in the presence of even a small amount of hexosediphosphate. This was tested first by Harden and Henley [1920] with zymin and later by Mayer [1927] with maceration extract. Raymond and Levene [1928] found that not only natural hexosephosphates (hexosediphosphate, Robison's and Neuberg's monophosphates) but also synthetic phosphates (3-glucose-, 1-fructose-, 3-fructose- and dihydroxyacetone-phosphates) had a marked effect in reducing the period of induction with zymin in large volumes of sugar solution, although hexosediphosphate greatly exceeded all the others.

Neuberg [1918] pointed out a remarkable effect of a number of aldehydes and other hydrogen acceptors in accelerating fermentation with maceration extract. These observations were confirmed by Harden and Henley [1920 and 1921, 1] with zymin.

Recently Harden [1925], and subsequently Harden and Macfarlane [1928], made the interesting observation that the period of induction in fermentation by zymin in large volumes of sugar solution was appreciably reduced by the addition of various inorganic and organic salts.

In the present paper the effect of inorganic and organic salts on the period of induction in the fermentation by dried yeast (prepared from the bottom type beer yeast in Japan) in the presence of phosphate has been studied in order to ascertain which salt would be more potent in reducing the period of induction, and what influence would be exerted on the period of induction when a mixture of salts is employed.

Methods and results.

The apparatus described by Harden, Thompson and Young [1910] was used. The fermentations were carried out at 25° by a suitable amount of dried yeast (about 0·8 g.) with 20 cc. of the various salt solutions containing

2 g. glucose, Na_2HPO_4 (0·04 *M*) and 0·2 cc. toluene, after saturation with CO_2. The amount of CO_2 evolved was observed at intervals of 10 minutes. The p_H values of the mixtures after saturation with CO_2 were determined colorimetrically in the same manner as was described by Biilmann and Katagiri [1927]. The p_H values were found to be 6·3, and no difference in p_H on the addition of the salts could be detected.

For the period of induction, the intersecting point of the two tangents to the curves of normal fermentation and of the initial slow fermentation was chosen as shown in Fig. 1.

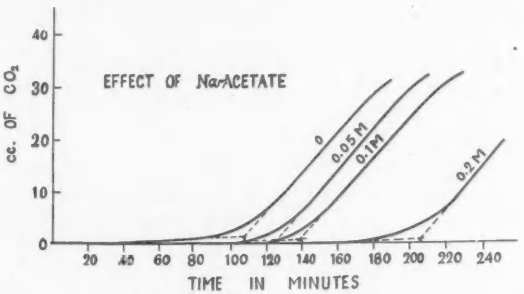

Fig. 1.

In order to allow for variation in the period of induction according to the sample of dried yeast, a fermentation was always carried out in the presence of 0·04 *M* phosphate without any other salt. The standard induction period under these circumstances was taken as 100 minutes. When the observed period had not greatly differed from this, the ratio of the two numbers was found and used as a factor to correct all the observations of the series to the standard.

When the sample of dried yeast had a considerably different fermentative power, the amount of dried yeast taken in each series was determined by a preliminary test so as to obtain nearly the same standard induction period (100 minutes). This amount of dried yeast varied from 0·75 to 1·0 g.

In order to test the validity of this method a series of experiments was made with 0·75, 0·8 and 1·0 g. of very different samples of dried yeast (Table I, Exps. 1, 2 and 3).

Table I.

Concentration of Na acetate (*M*)	Induction period (minutes)			Relative induction period		
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
0	73	90	108	100	100	100
0·025	81	100	123	111	111	114
0·05	93	120	128	128	133	119
0·1	95	127	139	130	141	129

It will be seen that the relative values agree well, although the actual values were, in each case, considerably different.

The results of the experiments which are given in Tables I–VI show the average values of more than two duplicated observations. No remarkable difference in the rate of evolution of CO_2 during the rapid fermentation, in each series of experiments, could be detected. However, when still higher concentrations of the salts than the maximum concentration shown in the tables were employed, a reduced rate of fermentation was always observed, as well as a longer period of induction, as was expected from the observations of Harden and Henley [1921, 2].

Effect of sodium salts.

(*a*) *Organic salts.* Table II (A) shows the effect of the salts of fatty monobasic and hydroxy-acids.

Table II (A). *Relative induction period with monobasic acids.*

Concentration of Na salt (*M*) ...	0·025	0·0375	0·05	0·075	0·1	0·2
Formate	98	—	96	—	105	147
Acetate	114	—	119	—	129	178
Propionate	112	123	138	207	>250	—
Butyrate	104	—	121	—	>180	—
*iso*Butyrate	94	—	115	—	136	—
Glycollate	181	—	196	—	>260	—
Lactate	116	—	150	—	215	—
β-Hydroxybutyrate	102	—	114	—	186	—

It will be seen that the period of induction is prolonged by the addition of these salts and the effect is more potent as the concentration of the salts increases, except formate and *iso*butyrate; with these a slightly reduced period is observed at lower concentrations. However, the observed effects are not the same: among the simple fatty acids formate has the least, while propionate has the greatest effect on the period of induction.

The effect of the hydroxy-acids is in each case greater than that of the corresponding fatty acid. The order of the potency of the effect of hydroxy-acids is inversely proportional to their molecular weights, while in the case of fatty acids no such simple relation could be observed.

Table II (B). *Relative induction period with polybasic acids.*

Concentration of Na salt (*M*) ...	0·00625	0·0125	0·025	0·05
Succinate	118	166	198	>290
Fumarate	119	182	>280	—
Malate	128	153	>230	—
Tartrate	155	200	>280	—
Citrate	270	>300	—	—

It will be seen in Table II (B) that the effect of succinate, fumarate, malate, tartrate and citrate is in each case greater as the concentration increases. The shortest induction is observed with succinate and the longest with citrate. Fumarate, malate and tartrate produce nearly equal effects.

The hydroxy-acids are more potent than the corresponding fatty acids, and the di- and tri-basic acids have a greater effect than the monobasic acids

in prolonging the induction with dried yeast in presence of 0·04 *M* sodium phosphate and sugar. This variation in the magnitude of the effect on the induction period is probably due to the specific nature of the salts, that is, to the nature of their ions.

(*b*) *Inorganic salts.* In order to ascertain the effect of inorganic anions, experiments were carried out with NaCl, NaI, $NaHCO_3$, Na_2SO_4. The results are given in Table III.

Table III. *Relative induction period.*

Concentration of salt (*M*) ...	0·00625	0·0125	0·025	0·05	0·1	0·2
NaCl	97	100	109	140	272	>300
NaI	136	>215	—	—	—	—
$NaHCO_3$	123	134	138	213	>310	—
Na_2SO_4	114	124	131	190	>250	—

It will be seen that these salts produce an effect similar to that of the Na salts of organic acids. The period of induction is again prolonged by the addition of these salts, and the effect is greater as the concentration of these salts increases. The only exception is NaCl, with which a slightly reduced induction period is observed at the lowest concentration.

When the relative induction periods are plotted against the concentration of Na^+ of the salts added, the curves in Fig. 2 show the relative potency of the effect of their anions on the period of induction.

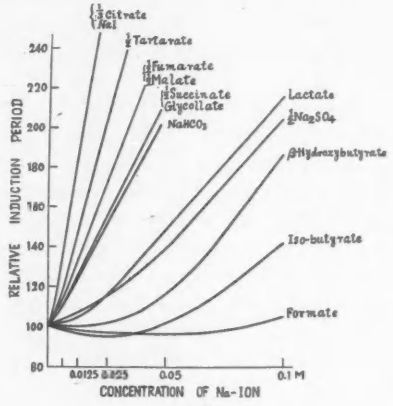

Fig. 2.

It will be seen that the iodide ion produces the greatest, and the formate ion the least prolongation of the period of induction.

Effect of K, Mg, NH_4 and Ca salts.

In order to investigate the effect of cations and to obtain further evidence of the specific effect of anions on the period of induction, experiments were made with various salts (Table IV).

Table IV. *Relative induction period.*

Concentration of salt (*M*) ...	0·00625	0·0125	0·025	0·05	0·1	0·2
KCl	88	90	82	89	110	177*
KI	109	104	177	>220	—	—
KBr	64	38	34	30	26	48
KNO_3	110	109	133	150	207*	>250
K_2SO_4	67	47	41	35	48*	84*
$MgCl_2$	—	85	81	73	91	213*
$MgSO_4$	—	67	47	29	25	37*
$CaCl_2$	—	102	110	>210	—	—
NH_4Cl	89	66	58	60	61	106
$(NH_4)_2SO_4$	15	19	19	23	32*	59*
NH_4 acetate	22	17	13	14	14	13
NH_4 lactate	32	22	15	15	14	22
NH_4 succinate	22	20	19	27	32	44*

* Represents the point at which a noticeably diminished fermentation rate is observed.

It will be seen that KI and KNO_3 produce an effect very similar to that of NaI; no reduction in the period of induction is observed at any concentration of these salts and the period is prolonged as their concentration increases. However, with KCl a reduced period is observed at the concentrations of 0·05–0·00625 *M*; the period of induction is gradually reduced until the concentration reaches 0·025 *M* and is then prolonged as the concentration increases. K_2SO_4 is more effective and KBr still more effective than KCl in reducing the period of induction. With these two salts the period is at first very rapidly, then very slowly reduced until the concentration reaches the optimum, after which the period is gradually prolonged as the concentration of these salts increases.

With both magnesium and ammonium salts (chloride and sulphate) a shorter period of induction is observed when these are compared with the corresponding potassium salts, and ammonium salts are more effective than magnesium salts in reducing the period. Among all the salts, $CaCl_2$ produces the greatest effect in prolonging the period of induction.

These observations show on one hand the specific effect of cations, and on the other that of anions. It is clearly seen, for example, in Fig. 3 in which the relative induction periods are plotted against the concentration of chloride ion, that the calcium ion produces the longest and the ammonium ion the shortest induction period. $\frac{1}{2}\,Ca^{++} > Na^{+} > K^{+} > \frac{1}{2}\,Mg^{++} > NH_4^{+}$.

Tables II, III and IV show that this order does not differ greatly when the sulphates are compared, and organic NH_4 salts (acetate, lactate and succinate) are much more effective than Na salts in reducing the period of induction.

The interpretation of this remarkable effect of ammonium salts is not very clear, but it is interesting to note that no specific effect of ammonium ion, as compared with sodium ion, is observed on the rate of normal fermentation of English living yeast [Katagiri, 1926].

As regards the specific effect of anions, it follows from the results in

Tables III and IV that the iodide ion produces the longest and the bromide ion the shortest induction period, the order of the anions being as shown below:

$$\begin{aligned} NaI > NaHCO_3 > NaCl &> \tfrac{1}{2}Na_2SO_4, \\ KI > KNO_3 > KCl &> \tfrac{1}{2}K_2SO_4 > KBr, \\ MgCl_2 &> MgSO_4, \\ NH_4Cl &> \tfrac{1}{2}(NH_4)_2SO_4. \end{aligned}$$

No direct comparison of these results with those of Harden and Macfarlane [1928] is possible, since the conditions of the experiment were different from theirs, dried yeast being used instead of zymin and an addition of 0·04 *M* phosphate being made. However, the results obtained with KCl, NH_4Cl, K_2SO_4 and $MgSO_4$ coincide with their observations that the induction period is reduced from 60 to 20 minutes in the presence of these salts, although the optimum concentrations of these salts are not quite identical.

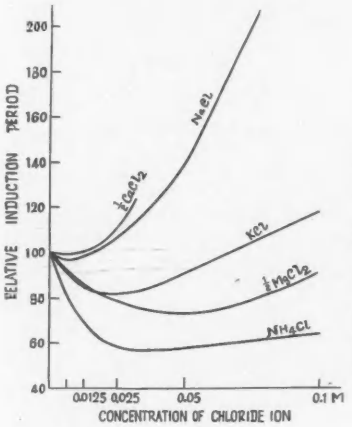

Fig. 3.

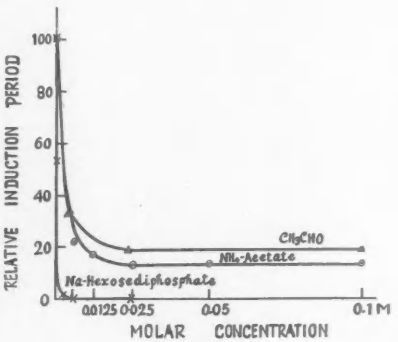

Fig. 4.

Thus it is concluded that among all the salts employed, NH_4 acetate produces the greatest effect in reducing the period of induction. When its effect is compared with those of Na hexosediphosphate and acetaldehyde, it will be seen (Fig. 4) that NH_4 acetate is a little more potent than acetaldehyde in reducing the period of induction, although Na hexosediphosphate is most effective.

Effect of salt mixtures.

In order to ascertain the combined effect of anions or cations it is necessary to keep the concentration of the accompanying ionic components constant.

Table V.

(A) Mixture of Na_2SO_4 and $(NH_4)_2SO_4$ in the presence of 0·04 *M* Na_2HPO_4.

Na_2SO_4 (*M*)	$(NH_4)_2SO_4$ (*M*)	Relative induction period	NH_4^+/Na^+
0	0·1	31	5
0·025	0·075	36	1·15
0·05	0·05	43	0·56
0·075	0·025	45	0·22
0·09	0·01	57	0·077
0·095	0·005	88	0·037
0·1	0	>250	0

(B) Mixture of K_2SO_4 and KNO_3 in the presence of 0·04 *M* Na_2HPO_4.

K_2SO_4 (0·5 *M*)	KNO_3 (*M*)	Relative induction period	$\frac{1}{2}\ SO_4''/NO_3'$
0·1	0	37	∞
0·075	0·025	51	3
0·05	0·05	66	1
0·025	0·075	128	$\frac{1}{3}$
0	0·1	219	0

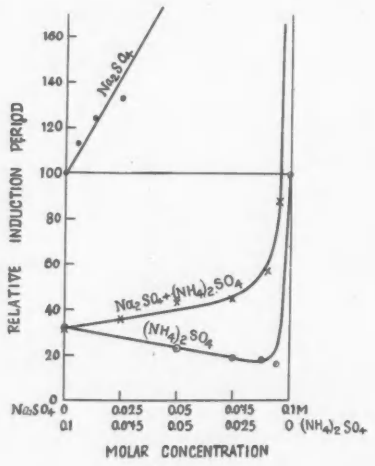

Fig. 5.

It will be seen from Table V (A), in which mixtures of Na_2SO_4 and $(NH_4)_2SO_4$ in various proportions are taken and the total concentration of sulphate is kept constant, that the period of induction is reduced as the concentration of NH_4^+ increases. When these values are compared with those of the individual effect of these salts, it will be seen (Fig. 5) that NH_4^+ is more potent than Na^+ in controlling the period of induction. In the presence of a small amount of NH_4^+ (0·01 *M*) the period of induction is remarkably reduced from the expected period (from about 200 to 57). The interpretation of these results is not very clear, but it is probably due to an antagonistic effect of NH_4^+ to Na^+.

When mixtures of K_2SO_4 and KNO_3, in which the total concentration of K^+ is kept constant, are employed, the sulphate ion exerts an antagonistic effect on the nitrate ion, although the effect is not so great as was found with NH_4^+ and Na^+ (Table V (B)).

In discussing the combined effect of Na^+ and NH_4^+, care should be taken to eliminate the effect of the sodium phosphate previously added. With this object, experiments were carried out with a mixture of NH_4 and Na phosphates in which the total concentration of phosphates was kept constant.

Table VI.

Mixture of Na_2HPO_4 and $(NH_4)_2HPO_4$.

(A) 0·04 *M* phosphate:

Na_2HPO_4 (*M*)	$(NH_4)_2HPO_4$ (*M*)	Relative induction period	NH_4^+/Na^+
0·04	0	100	0
0·038	0·002	33	0·05
0·036	0·004	21	0·11
0·032	0·008	18	0·25
0·02	0·02	11	1
0·008	0·032	9	4
0	0·04	4	∞

(B) 0·08 *M* phosphate:

Na_2HPO_4 (*M*)	$(NH_4)_2HPO_4$ (*M*)	Relative induction period	NH_4^+/Na^+
0·08	0	>300	0
0·06	0·02	33	$\frac{1}{3}$
0·04	0·04	28	1
0·02	0·06	21	3
0	0·08	18	∞

It will be seen in Table VI (A) and (B) that the period of induction is reduced in each case (0·04 *M* and 0·08 *M* phosphate) as the concentration of NH_4^+ increases.

When the relative induction periods are plotted against the ratio of NH_4^+ and Na^+ (Fig. 6), the curves are of much the same character. A hyperbolic relation can be supposed to exist between the relative induction period (= *T*) and the ratio of the two ions (= *R*); thus

$$T \times R^{\frac{1}{n}} = \text{constant},$$

where *n* is a constant according to the nature of the salt; with 0·04 *M* phosphate $n = 3$ and with 0·08 *M* phosphate $n = 4{\cdot}86$.

If the ratio of NH_4^+ and Na^+ is based on the total concentration of Na ion (added Na_2SO_4 plus sodium phosphate previously added), it will be seen (Fig. 6) that the curve of 0·1 *M* sulphate + 0·04 *M* phosphate (which is incorporated in Table V (A)) is again of much the same character, and the above equation is also applicable when $n = 5$ is chosen as the constant for this mixture. It will be considered from these results that a simple antagonistic relation exists between the two cations NH_4^+ and Na^+; NH_4^+ acts in opposition to Na^+ in reducing the period of induction.

Fig. 6 shows that this kind of antagonism is also found between the two anions of sulphate and nitrate.

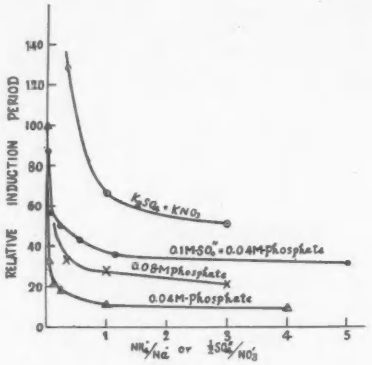

Fig. 6.

Summary.

1. Fermentation by dried yeast in the presence of 0·04 *M* phosphate was observed with various concentrations of inorganic and organic salts.

2. The period of induction was found to vary with the concentration and with the specific nature of the salt; both the cation and the anion.

3. The order of the potency of the salts in prolonging the period of induction was found to be $\frac{1}{2}\,Ca^{++} > Na^{+} > K^{+} > \frac{1}{2}\,Mg^{++} > NH_4^{+}$ with chlorides and sulphates, and $I' > Cl' > \frac{1}{2}\,SO_4''$ with Na and K salts.

4. It was found that NH_4 salts (acetate, lactate, succinate and sulphate) were nearly as potent as acetaldehyde in reducing the period of induction, although they were less potent than hexosediphosphate.

5. The combined effect of the two ions was examined. NH_4^{+} produced a remarkable antagonistic action to that of Na^{+}, and sulphate ion a slight antagonistic action to that of nitrate ion.

6. A hyperbolic relation may be supposed to exist between the induction period and the ratio of the concentrations of the two ions.

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LXXVI. BLOOD-FAT[1].

I. PREPARATION AND GENERAL CHARACTERISTICS.

By HAROLD JOHN CHANNON
and GEORGE ARTHUR COLLINSON.

From the Department of Experimental Pathology and Cancer Research, University of Leeds.

(*Received June 29th, 1929.*)

Until adequate information regarding the chemical nature of the substances in which fatty acids are present in blood-fat is available, little progress can be made with the problem of the part which phosphatides, cholesteryl esters or other compounds may play in the transport of fat to the tissues. That such information is lacking is demonstrated by the following brief reference to the literature on blood-fat.

The earlier results record figures for the total fatty acids determined in many cases by use of the modification of the Kumagawa-Suto saponification method [1908] devised by Shimidzu [1910]. Latterly, an endeavour has been made, notably by Bloor [1915] and Bloor, Pelkan and Allen [1922] to obtain more detailed information by micro-analysis, in which estimations of total fatty acids, free and combined cholesterol and ether-soluble phosphorus have been carried out; and in this way evidence has been brought to bear on the question of the distribution of the fatty acids in the different combinations in which they may occur in blood. Consideration of the many results recorded shows, however, that very wide variations in any given value exist, and in some cases the findings are directly opposed. It is true that some of these variations may be accounted for by the fact that insufficient attention has been paid to the state of absorption of the animal whose blood has been investigated, but the impression remains that many of the results are due to the employment of methods ill-suited to the problem.

Although this abundance of quantitative data exists, there is a dearth of information concerning attempts to prepare substances in any state of purity from blood-fat. From time to time, claims to have obtained individual substances from blood have been made, such as those of Hürthle [1895] to have

[1] It will be shown during the discussion of the results that glycerides are virtually absent from the blood of the fasting ox and that almost all the material consists of phosphorus-containing compounds of fatty acids, together with cholesterol and its esters. Although "fat" is thus absent, we shall refer to the ether-soluble material throughout as "blood-fat" for the sake of ease of description, for the generic word "lipide" proposed by the International Congress of Applied Chemistry and used by Bloor [1925] has not found acceptance in this country, and the variety of terms used by other workers is confusing not only to the reader but to those engaged in the same field.

prepared the oleyl, palmityl and stearyl esters of cholesterol from serum, and of Bürger and Beumer [1913], who prepared sphingomyelin and kephalin from the stromata of red cells. Bloor [1923] prepared the fatty acids of plasma and corpuscles and studied their iodine values, and the same author [1924] prepared the fat from quantities of plasma up to 2 litres. To an ether solution of this fat he added excess of acetone, hydrolysed the precipitate and studied the iodine values of the fatty acids so obtained. The acetone-ether-soluble fraction was fractionally crystallised from alcohol and each fraction was then saponified and the iodine values of its constituent fatty acids determined. As a result of these latter researches, Bloor suggests that the unsaturated cholesteryl esters present are either the linoleate or a mixture of the oleate with the esters of acids more unsaturated than oleic acid. It will be remembered also that the ether-soluble phosphorus of blood, of which so many estimations have been made, is usually recorded as lecithin, although little information other than that just mentioned is available regarding its nature, obviously on account of the difficulty of working on an adequate scale. Our object, therefore, was to attempt to prepare blood-fat in a quantity sufficient to enable us to submit it to chemical examination with a view to obtaining more definite information as to the compounds in which phosphorus and cholesterol occurred and in the hope that such a study might result in our being able to express an opinion as to the validity of the results obtained by the many micro-methods of blood-fat estimation.

Another question which we have borne in mind is whether calcium phosphatidate, which may be regarded as the parent acid of lecithin and kephalin [Channon and Chibnall, 1927], is present in blood. Kephalin preparations usually contain calcium which can be removed by shaking with mineral acid [Diaconow, 1867; Thudichum, 1884; Parnas, 1909]. Calcium phosphatidate, like kephalin, is insoluble in alcohol and its calcium may be similarly removed, so that it is possible that this substance may be responsible for the calcium which usually contaminates kephalin preparations. If calcium phosphatidate is present in blood, the rise in ether-soluble phosphorus of the blood after a fatty meal, which has been observed and investigated by Bloor [1915], might be due to that substance which is a possible intermediate in the synthesis of lecithin and kephalin from the absorbed fat. Further, the study of the nature of blood-fat in a fasting animal may throw light on the hypothesis of Leathes and Raper [1925], who consider that the fatty acids of adipose tissue may be desaturated in the liver and that after being converted into highly unsaturated liver phosphatides they may be transported to the tissues in this form.

Before attempting to submit a large amount of blood-fat to fractionation into its various constituents, it was considered wise to prepare a number of samples and to make determinations of the fat constants and of phosphorus and nitrogen on the material in order to gain some idea as to its constancy of composition or otherwise, and to obtain some guidance for the treatment of the large preparation.

Preparation of blood-fat.

Our object in the preparation has been to obtain as much fat from blood with as little alteration of its original character, either by oxidation or by enzyme action, as possible. Blood contains rather less than 0·5 g. of fat per 100 cc., some of which is held by the protein in a form which prevents it from being easily removed. The difficulties encountered in attempting to remove this fatty material from so complex a tissue as blood are such that any method must necessarily be a compromise at many points, and one of them is well illustrated by the work of Shimidzu [1910], who found that the well-known saponification method of Kumagawa and Suto [1908] for the estimation of the total fatty acids in tissues cannot be applied to blood, a finding which we confirm. Shimidzu, therefore, devised a method in which blood is run into several volumes of alcohol and, after shaking, the mixture is allowed to stand for some hours. It is then filtered, and the protein residue is extracted for 6–8 hours by exposure to the vapours of boiling alcohol by suspension in a thimble over the surface of the solvent in a flask fitted with a reflux condenser. This alcohol extract is united with the original alcohol filtrate and the mixture is saponified and the fatty acids prepared by the Kumagawa and Suto method. In spite of this rigorous treatment, some material which on hydrolysis gives rise to fatty acids still remains in the protein residue, and these fatty acids, which can only be recovered by hydrolysis of this residue, amount to not more than 5 % of the total [Mayer and Schaeffer, 1913]. The material obtained by the alcohol extraction (prior to saponification) by the Shimidzu method is usually dark red in colour and contains a large proportion of substances which do not contain fatty acids but which are soluble in fat solvents, possibly because of the presence of the blood-phosphatides. Hence such an extraction method, although of great use for estimating the total fatty acids in blood, cannot be utilised for the preparation of blood-fat on account of the high proportion of extraneous material introduced, the length of time necessary for extraction with likelihood of consequent oxidation, and the impossibility of extracting large amounts of blood-protein in an atmosphere of hot alcohol vapour.

The alternative methods for obtaining the blood in a suitable form for extraction by fat solvents are precipitation by a solvent miscible with water or direct drying with suitable precautions against oxidation. No satisfactory method of direct drying being found, we were compelled to adopt a precipitation procedure in which we used alcohol.

Little advantage is gained by adding more than three volumes of alcohol to each volume of blood, for the increase in the percentage of alcohol in the filtrate by the addition of one or more volumes beyond three is relatively insignificant. It was decided to submit the protein residue to extraction with ether and not with alcohol as in the Shimidzu method, in order to avoid obtaining in the extract much material not of fatty nature to which reference

has been made. Such ether extraction will leave a definite amount of the fat unextracted, but a compromise at this point in favour of obtaining a less contaminated product which can be more rapidly prepared appeared desirable. The alcohol filtrate from the precipitation was evaporated to dryness and the dry residue also extracted with ether, and the extracts from the two fractions were combined. The relative amounts of material extracted from the protein residue and the alcoholic filtrate depended on the time during which the mixture of blood and alcohol was allowed to stand, but usually up to 80 % of the final material was obtained from the alcohol filtrate. Consideration was also given to the question as to whether it would be worth while to draw off the oxygen of the blood by exposure in a vacuum before precipitation in order to minimise oxidation (the solubility of oxygen in alcohol is about 20 times that in water). Accordingly experiments were carried out in which blood was treated by the method outlined above before and after removal of its oxygen, and iodine values were determined on the samples of fat obtained. Typical of these may be quoted the results of one experiment in which the iodine value of the fat of the blood directly precipitated was 58·2, and that of the same blood which had been previously sprayed into an evacuated flask was 58·9. In view of the fact that we were dealing with large quantities of blood, this difference was not considered sufficiently great to render it essential to remove the oxygen before treating the blood with alcohol. The material obtained by ether extraction of the protein residues was in all cases a waxy mass, golden yellow in colour, while that from the ether extract of the residue from the alcohol filtrates was reddish brown and contained much foreign material which was found to consist of nitrogenous substances. Since the bulk of the fat was obtained in the latter fraction, no useful purpose was served by keeping the two fractions apart and they were accordingly mixed.

Method.

Ox blood was received into oxalate at the slaughter-house and run into three volumes of alcohol within 30 minutes of being drawn. As the animals usually receive no food for 24 hours before slaughter, the fat of such blood may be regarded as at fasting level. The mixture was then vigorously shaken and allowed to stand for varying times, usually 0·5 to 2 hours. In the case of the 40 l. quantity, it was necessary to precipitate large volumes of blood at one time, and these stood in alcohol for varying times up to 24 hours. After standing, the mixture was filtered on a Büchner funnel. The residue so obtained needed considerable pressing before it became reasonably solid, and even then it was found to contain large amounts of solvent, for the volume of filtrate was never greater than 1·2 times the volume of alcohol added. The residue was then extracted in a Soxhlet apparatus until the ether extract was colourless. The original alcohol filtrate was evaporated to dryness in the water-bath at low pressure: after the volume had been rapidly reduced to about one-half, the solution frothed excessively. This frothing could only be

overcome by lowering the temperature of the water-bath at this stage and reducing the water pressure to the minimum necessary to maintain a vacuum, and evaporation was extremely slow. When the volume was reduced to about 10 % of the original extract frothing ceased and the evaporation could be continued as before. The last stages of the evaporation were also slow, for the fatty material which separated at the half-way stage then formed an emulsion, and only by raising the temperature of the water-bath to 80° could the water be effectively removed. In the final stages, absolute alcohol was repeatedly added to ensure efficient removal of the remaining water. Evaporation of the filtrate obtained from 1 litre of blood took 7–10 hours. The ether extract from the protein residue was also evaporated to dryness. The two residues were then washed out with ether into a stoppered cylinder and allowed to stand overnight. The solutions were filtered into weighed vessels, the solvent was removed, and a weight obtained after drying on the water-bath *in vacuo*. The material so obtained is usually reddish brown in appearance and, when warm, transparent; when cold it has the appearance (apart from colour) of a mixture of phosphatide and cholesterol, and is a sticky translucent mass. It contains, as we shall see later, a considerable amount of materials which are not truly ether-soluble. Its solution in ether is slightly fluorescent, and is usually turbid.

Determination of constants.

Aliquot portions of the ether solutions were used for duplicate determinations as set out in Table I. Nitrogen was determined by the micro-Kjeldahl method on 20–25 mg. quantities. The low phosphorus content (1–2 %) of the material would entail the employment of quantities of the order of 0·3 g. for a gravimetric estimation as magnesium pyrophosphate and we have been compelled in the routine determination of constants, and in the subsequent fractionation of the large batch of material on which a large number of phosphorus determinations has been necessary, to use the quinol-sulphite colorimetric method after oxidation of 8–12 mg. portions with sulphuric acid and "perhydrol." Cholesterol was determined by the Windaus [1910] digitonin method. The saponification values were determined on 0·5 to 1·5 g. portions by heating with 0·25 *N* (approx.) alcoholic potassium hydroxide for 4 hours. After titration, a considerable part of the alcohol was removed, and after being made strongly alkaline with potassium hydroxide the solution was poured into water in a separating funnel and exhaustively extracted with ether. The ether extract, after thorough washing with water, was evaporated to dryness and resaponified for one hour with sodium ethylate in alcoholic solution to ensure the complete hydrolysis of cholesteryl esters. After dilution with water, the mixture was again ether-extracted. The ether extract was thoroughly washed with water, and evaporated to dryness in a weighed flask. In this way the unsaponifiable fraction was obtained. It was, in all cases, a hard crystalline mass, of deep orange colour, the colour being

due to the lipochrome pigments carotene and xanthophyll: on this material total cholesterol was determined. The soap solutions from both saponifications, together with the washing of the ether solutions, were united and acidified with hydrochloric acid, and then extracted with ether. Difficulty has been encountered at this point, because acidification always causes the precipitation of a certain amount of slimy material which is insoluble in ether, and forms an emulsion at the ether-water interface. It has been our practice to leave this material suspended in the funnel with the first ether extract, and then to add to this ether extract the four or five successive ether extracts of the aqueous acid phase. The combined extracts were washed with water, until free from mineral acid, and then filtered. The insoluble material to which reference has just been made renders this filtration tedious. As to its nature we have no information, but such emulsions are encountered in the Shimidzu method of estimating blood-fat as pointed out by Mayer and Schaeffer [1913] and seem unavoidable with this type of material. The ether solutions of the fatty acids were evaporated, and dried *in vacuo* on the water-bath. The residues were dissolved in warm light petroleum (B.P. 40–60°) and allowed to stand overnight for the so-called "resinous" materials to separate out. After further filtration and washing of the residues, the light petroleum extracts containing the fatty acids were evaporated to dryness and weighed. Determinations of the iodine values (Wijs) and the molecular weight by titration were then carried out. The results are recorded in Table I.

Table I. *Analysis of the ether extract.*

(All figures are expressed as a percentage of the ether extract.)

Sample No. ...	1	2	3	4	5	6	7	8	9	10	11	12	Mean
Vol. of blood used, cc.	400	300	40,000	1200	1000	1000	350	350	350	350	500	400	—
Yield, g./l.	4·60	5·11	3·97	—	4·73	4·19	4·72	4·90	4·56	5·47	6·01	6·65	4·99
N	2·48	2·09	2·39	2·15	2·39	2·99	2·37	2·38	1·96	2·11	2·44	2·46	2·35
P	1·51	1·57	1·57	1·65	1·62	1·63	1·66	1·64	1·56	1·57	1·37	1·52	1·57
Unsap. matter	31·55	32·08	31·81	34·85	26·44	27·16	27·83	32·29	31·45	34·45	29·16	30·14	30·76
Free cholesterol	17·74	16·04	17·04	22·46	14·40	15·68	17·41	15·80	17·3	15·1	13·50	12·88	16·27
Cholesterol as ester	8·22	6·96	6·43	7·88	8·85	7·14	8·59	7·18	10·39	14·08	13·21	15·50	9·53
Unsap. matter other than cholesterol	5·59	9·08	8·34	4·51	3·19	4·34	1·83	9·31	3·76	5·27	2·45	1·76	4·95
Fatty acids	36·0	30·0	32·5	34·8	37·7	34·9	37·9	30·7	38·3	35·5	32·7	35·3	34·7
Iodine value	—	—	100	91·6	87·8	89·3	87·5	94·4	92·9	97·3	116·2	117·7	97·5
Mol. wt.	—	—	294	298	298	304	311	294	310	294	307	305	301

Discussion.

The proportion of fat extracted.

The figures in Table I show that the mean average phosphorus content of 12 samples of fat is 1·57 % P (extreme values 1·37, 1·66 % P), which corresponds to 7·78 mg. P per 100 cc. blood. Determinations of the ether-soluble phosphorus of 15 samples of ox blood gave a mean value of

9·8 mg./100 cc., which suggests that the method used has resulted in the extraction of 80 % of the ether-soluble phosphorus present. In order to gain further information as to the proportion of material obtained, the fat was prepared from a number of samples of blood, and at the same time determinations of the sum of the amounts of unsaponifiable matter and fatty acids were made on 50 cc. portions of blood by the Shimidzu [1910] method, save that the protein residues were not hydrolysed. In the calculations of the percentage yield allowance has been made for the fact that up to a further 5 % of material may be obtained from the hydrolysis of these residues. The results are recorded in Table II.

Table II. *Fatty acids and unsaponifiable matter extracted, calculated on the yields obtained by the Shimidzu method* [1910].

(Results expressed in mg./100 cc. blood.)

Fatty acids	179·1	150·4	174·9	194·4	212·0	239·0
Unsaponifiable matter	152·4	136·3	143·5	189·0	170·4	180·4
Total	331·5	287·6	318·4	383·4	382·4	419·4
Fatty acids and unsap. matter (Shimidzu)	369·6	361·4	365·2	433·8	430·0	515·6
Percentage yield	85·2	75·3	82·8	84·0	84·5	77·2

The mean figure for the yield expressed as a percentage of that of the Shimidzu method is 81·5 %. This figure is similar to that obtained for the proportion of phosphorus extracted, and it seems justifiable therefore to consider that the fat extract is probably fairly representative of the whole.

One further point needs mention. With small quantities of blood, ether extraction of the protein residue is readily accomplished. With larger quantities, such extraction is more difficult, and although every care was taken to see that thorough extraction was obtained, it is possible that some of the variations in yield may be accounted for to some extent by this fact. This remark applies in particular to the 40 l. quantity.

We have discussed the method of preparation and the question of the proportion of fat extracted somewhat fully because it was the one finally adopted, and because that used by Bloor [1924] in the simpler problem of preparing fat from plasma seemed to us unsatisfactory.

Consideration of Bloor's results, in which 16 samples of ox blood-plasma varying in volume from 500 to 2880 cc. were used, shows that the yield of phosphatide fatty acids varies from 0·0021 to 0·0799 g./100 cc., while in 5 cases apparently no phosphatide fraction was obtained, for the yields of fatty acids are not recorded. These results may be calculated in terms of ether-soluble phosphorus (fatty acids = P × 18·26), and such calculation shows that the average yield of fatty acids from the phosphatide fractions corresponds to an extraction from the plasma of 1·06 mg. of ether-soluble phosphorus per 100 cc. (extreme values 0·11 and 4·37 mg.), whereas the average figure obtained by us in direct determination of the ether-soluble phosphorus of 12 samples of ox blood-plasma is 6·62 mg./100 cc. Hence the method used by Bloor appears to have resulted in his obtaining an average

yield of not more than 16 % of the phosphatide fatty acids present in the plasma. Bloor also points out that early in his work it was discovered that the fat obtained contained free fatty acids which were removed and weighed. In this connection, analysis of the results from the four samples for which the data are most complete shows that in these four cases the material contained from 10 to 33 % of its content of fatty acids as free fatty acid, and these free fatty acids must have resulted from decomposition of some part of the fat during the preparation. Bloor comments on the low yield of fatty acids from the phosphatide fractions, and suggests that "insoluble residues are formed at certain points in the procedure which probably contained the altered phospholipoid; also, that in carrying out the various processes some of the lipoid was probably decomposed. The fact that a considerable amount of free fatty acid was always present in the lipoidal material, which would hardly have existed in the plasma itself, bears out the latter explanation." A material such as this, containing a large proportion of decomposition products, and only a small proportion of the phosphatide present in the plasma, which phosphatide may or may not be representative of the whole in the degree of unsaturation of its acids, appears to us to be unsatisfactory, and the conclusion drawn from its analysis open to doubt.

The fat prepared from blood by our method has never contained free acid, while it has been pointed out that its phosphorus content corresponds to an extraction of about 80 % of the ether-soluble phosphorus present in the blood.

In view of Bloor's experience with plasma, we submitted 25 cc. portions of 11 samples of plasma to the method which we have used for whole blood, in order to determine what proportion of the ether-soluble phosphorus was extracted, and whether the material contained free acid. The results are recorded in Table III. In no case did the extracted fat contain free acid. It is seen that about 80 % of the ether-soluble phosphorus present in the plasma has been obtained in the fat.

Table III.

(Plasma: ether-soluble phosphorus determinations and percentage of phosphorus extracted.)

													Mean
Ether-soluble P, mg./100 cc.	6·04	7·62	9·57	7·60	6·39	8·43	6·53	5·59	4·21	5·79	6·73	4·92	6·62
Wt. of fat, g./100 cc.	0·512	0·504	0·721	0·621	0·512	0·770	—	0·452	0·326	0·489	0·555	0·433	0·536
P as % of fat	1·03	0·91	1·19	1·08	0·92	1·02	—	1·04	0·89	0·91	0·87	1·01	0·99
P in fat as mg./100 cc. plasma	5·27	4·56	8·62	6·73	4·71	7·88	—	4·76	2·91	4·43	4·84	4·37	5·37
Percentage of P extracted	87	60	90	89	74	93	—	85	69	75	72	89	80

Absence of glycerides.

The initial observation to be made from the figures of Table I is that the sum of the average values of the total unsaponifiable matter (30·76 %) and of the fatty acids (34·69 %) is only 65·45 % of the material. This will be

accounted for in part by the fact that lecithin on hydrolysis yields but 70·3 % of its weight of fatty acids, but the greater part of the difference is due to the presence of extraneous nitrogen in the material. Before discussing this matter further, attention may be drawn to the amount of fatty acid which percentages of phosphorus and cholesteryl ester require[1], and which are set out in Table IV. It will be seen that there is, in general, fair agreement between the percentage of fatty acids found and those calculated on the basis of the phosphorus being present as lecithin and the cholesteryl ester as stearate[2].

Table IV. *Fatty acids.*

(Figures are expressed as a percentage of the fat.)

Sample No.	1	2	3	4	5	6	7	8	9	10	11	12
Phosphatide fatty acids (P × 18·26)	27·6	28·7	28·7	30·1	29·6	29·8	30·3	29·9	28·5	28·7	25·0	27·7
Fatty acids combined with cholesterol (cholesteryl ester × $\frac{284}{386}$)	6·0	5·1	4·7	5·8	6·5	5·2	6·3	5·3	7·6	10·3	9·9	11·4
Total	33·6	33·8	33·4	35·9	36·1	36·0	36·6	35·2	36·1	39·0	34·9	39·1
Found	36·0	30·0	32·5	34·8	37·7	34·9	37·9	30·7	38·3	35·5	32·7	35·3

If such calculations are justified, it is clear that there can be little glyceride present, a conclusion which the later fractionation of the fat from 40 l. of blood showed to be correct. In Table V are recorded the percentages of lecithin, cholesterol, cholesteryl ester and unsaponifiable matter other than sterol in the fat, what little glyceride there is present being neglected, the object being to gain an approximate figure for the percentage of non-fatty material present.

Table V.

(All figures are expressed as a percentage of the fat.)

Sample No.	1	2	3	4	5	6	7	8	9	10	11	12
Lecithin (P × 26)	39·22	40·78	40·78	42·85	42·07	42·34	43·11	42·59	40·52	40·78	35·82	39·48
Cholesterol	17·74	16·04	17·04	22·46	14·40	15·68	17·41	15·80	17·36	15·10	13·50	12·88
Cholesteryl stearate	13·89	11·76	10·86	13·30	14·95	12·06	14·51	12·13	17·54	23·77	22·32	25·00
Unsap. matter other than cholesterol	5·59	9·08	8·34	4·51	3·19	4·34	1·83	9·31	3·76	5·27	2·45	1·76
Total	76·44	77·66	77·02	83·12	74·61	74·42	76·86	79·83	79·18	84·92	74·09	79·12
Residue; not fat	23·56	22·34	22·98	16·88	25·39	25·58	23·14	20·17	20·82	15·08	25·91	20·88

[1] From this point onwards, calculations involving the phosphatide will be made as has been done by previous workers on the basis of stearyl-oleyl-lecithin (N = 1·74 %, P = 3·85 %, fatty acids = P × 18·26); the cholesteryl ester will be regarded as cholesteryl stearate, an assumption which will subsequently be shown to be sufficiently accurate for the purpose of calculation.

[2] As pointed out on p. 668, difficulty has been encountered in extracting the fatty acids from the acidified soap solution on account of the presence of emulsions. Another difficulty which we have failed to overcome is that on no occasion have we obtained fatty acids which appeared "clean," in spite of the fact that attempts were made to remove what appeared to be resinous materials by second and third treatments with light petroleum. The same difficulty has been encountered by Rosenthal and Trowbridge [1915] using the Shimidzu [1910] method. Hence we cannot feel confident that the figures recorded in the above table are more accurate than to within possibly 5 %. We are investigating this matter further.

Presence of excess of nitrogen.

The sum total of these constituents is never greater than 83 %, and up to 25 % of the material consists of nitrogenous substances, as is shown by the excess of nitrogen over what is required by the phosphatide present. In the following table, the nitrogen percentage in excess of what is required by the phosphorus content and a N : P ratio of unity is shown, together with the percentage of non-fatty material (from Table V); the nitrogen percentage of the latter has been calculated.

Table VI. *The excess nitrogen expressed as percentage of the fat.*

Sample No. ...	1	2	3	4	5	6	7	8	9	10	11	12
N required if P is calculated as lecithin	0·68	0·71	0·71	0·74	0·73	0·74	0·75	0·74	0·70	0·71	0·62	0·68
N found	2·48	2·09	2·39	2·15	2·39	2·99	2·37	2·38	1·96	2·11	2·44	2·46
Excess N	1·80	1·38	1·68	1·41	1·66	2·25	1·62	1·64	1·26	1·40	1·82	1·78
Residue not fat (from Table V)	23·56	22·34	22·98	16·88	25·39	25·58	23·14	20·17	20·82	15·08	25·91	20·88
N percentage in residue not fat	7·66	6·18	7·31	8·35	6·54	8·80	7·00	8·13	6·05	9·34	7·03	8·54

The fact that an alcohol extract of a tissue may yield phosphatide preparations which contain far more nitrogen than is required by the amount of phosphorus present was shown by MacLean [1912] in his investigations on horse kidney, where the alcohol extracts yielded phosphatide material having N : P ratios varying from 4 : 1 to 1·5 : 1. He found that this excess of nitrogen was due to the fact that in the presence of phosphatides certain unknown nitrogenous substances are carried into solution and act as though they possessed all the properties of the phosphatides themselves. They can be removed in one way only, namely, by precipitation of the phosphatide emulsion in water by the addition of acetone. The nature of the complex mixture of these nitrogenous materials which accompany the phosphatides is discussed at length by MacLean and Smedley-MacLean [1927] under the generic name carnithin, where it is shown that the average nitrogen content of various samples was 6 % nitrogen and that substances with a nitrogen content of 28·5 and 40 % have been isolated from the mixture. It may be wondered why no attempt was made to remove this material from the blood-fat. The reasons are that acetone precipitation of an aqueous emulsion of the material results, as would be expected, in considerable losses of material which remains in the aqueous phase. The process needs repetition, and further it was considered preferable to analyse the fat, contaminated as it was with this material, rather than to remove it and obtain a purer preparation which might not be representative of the whole. Attempts were made to obtain fat from blood which did not contain this excess nitrogen by the use of different solvents, but these were unsuccessful. That the nitrogen percentage of the non-fatty residue in our material is higher (7·58 %) than that recorded by MacLean (6 %) may be due to the fact that calculation has been made of the nitrogen

required by the phosphorus on the basis of a N : P ratio of 1 (lecithin and kephalin) whereas it will be shown subsequently that some part, at least, of the blood-phosphatide may be sphingomyelin (N : P = 2 : 1, fatty acids = P × 12), the presence of which in red cells Bürger and Beumer [1913] claim to have demonstrated. On the other hand, the high and variable contents of nitrogen in the different substances obtained from samples of carnithin show how complex a mixture it is, and hence a constant nitrogen content is not to be expected.

Fatty acids.

The average figures for the molecular weights and iodine values of the fatty acids are 301 and 97·5 respectively. Of the figures available concerning the iodine value of blood-fatty acids, mention may be made of the finding by Imrie [1915] of an iodine value of 73 in a case of lipaemia, and of the results of Boggs and Morris [1909], who found that the iodine value of the fatty acids of the blood of rabbits rendered lipaemic by bleeding varied from 105 to 134. Csonka [1918] obtained a value of 87 for the liquid fraction of the mixed fatty acids of normal human blood (48 % of the total fatty acids) which corresponds to a value of 41. Bloor [1923] found an average iodine value of 147 for the liquid fraction of the fatty acids from 16 samples of ox blood-plasma. The liquid acids constituted 74 % of the total acids which, therefore, had an iodine value of 109 as against 97·5 found by us for those of whole blood. The higher figures obtained by Bloor may be due to the fact that he removed from the acids during the lead salt separation about 15 % of material, the lead salt of which was insoluble in hot alcohol and which on decomposition yielded a residue of low iodine value. The weight of this material was not included in the calculation of the percentage of the total acids which the liquid fraction represented, and its inclusion would lower the iodine value of the mixed acids to about 100. There is little doubt that the iodine values of the blood-fatty acids are higher than those recorded in this paper, for the nature of the starting material and the lengthy operations involved in their preparation must result in some degree of oxidation occurring in spite of the precautions taken to avoid it. Further, as has been pointed out already (p. 671 note 2), we think that the fatty acids have been contaminated by some other material which would also tend to lower the iodine values. That such contamination may exist appears to be borne out by the fact that the average molecular weight of the fatty acids is 301 (stearic acid has M.W. 284). Although, as will be shown subsequently, we have obtained from blood what appears to be lignoceric acid (M.W. 368), the amount present does not seem to be sufficiently great to account for the high molecular weight of the blood-fatty acids.

We have discussed all results in terms of percentages of the fat itself, and for the more physiological considerations some of them have been converted into yields expressed as mg. per 100 cc. of blood in Table VII.

Table VII.

(All figures are expressed as mg./100 cc. blood.)

Sample No. ...	1	2	3	5	6	7	8	9	10	11	12
P	6·95	8·02	6·23	7·66	6·83	7·83	8·03	7·11	8·59	8·23	10·10
Fatty acids combined with P (P × 18·26)	126·9	146·4	113·8	139·9	124·7	143·0	146·7	129·9	156·8	150·3	184·5
Fatty acids combined with cholesterol	27·83	26·16	18·78	30·80	22·01	29·83	25·88	34·85	56·63	58·41	75·82
Total fatty acids	154·7	172·6	132·6	170·7	146·7	172·8	172·6	164·8	213·4	208·7	260·3
Total fatty acids found	165·6	153·5	129·0	178·3	146·2	178·8	150·4	174·7	194·2	196·4	235·0
Free cholesterol	81·62	81·96	67·64	68·13	65·69	82·15	77·43	78·89	82·6	81·14	85·66
Cholesterol as ester	37·82	35·56	25·53	41·86	29·91	40·54	35·18	47·37	77·00	79·39	103·0
Unsap. matter other than sterol	25·71	46·39	33·11	15·09	18·19	8·64	45·6	17·15	28·82	14·72	11·70

If allowance be made for the amount of material other than fat present in the ether extract, it may be calculated that the yield of fat varies from 3·05 to 5·34 g./l. with a mean value of 3·97 g./l. The yield of fatty acids varies from 1·29 to 2·35 g./l., giving an average figure of 1·73 g./l., while the corresponding figures for the unsaponifiable fraction are 1·138 and 2·004 g./l., and 1·520 g./l. respectively. Thus the sum of the yields of unsaponifiable matter and fatty acids is 3·267 g./l., and the unsaponifiable fraction thus constitutes 46·8 % of this material, with values varying not greatly from the mean, viz. 42·3 to 49·5. Bloor [1923] obtained for the sum of the unsaponifiable matter in fatty acids an average yield of 3·01 g./l. from plasma, and found that the unsaponifiable matter constituted 43 % of this total. The unsaponifiable matter contains on the average 84 % of sterol precipitable by digitonin.

Although the saponification values of the fats were determined, they have not been recorded because the presence of the nitrogenous material already discussed vitiates them. They were carried out in the hope that if any considerable amount of glyceride were present in any sample of fat it would make itself obvious by raising the saponification value obtained. The mean of these values was 117.

Summary.

1. A method is described by which 80 % of the fat present in blood may be extracted. The material so obtained contains considerable quantities of nitrogenous substances.

2. Fat has been prepared from 12 samples of ox blood at fasting level, and a study has been made of its composition by determination of the fat constants and of phosphorus and nitrogen.

3. The amount of fatty acid present varies little from that required if the phosphorus of the fat be calculated as lecithin, and the cholesteryl ester as cholesteryl stearate, which suggests that glycerides are not present in appreciable quantity.

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LXXVII. THE UNSAPONIFIABLE FRACTION OF LIVER OILS.

V. THE ABSORPTION OF LIQUID PARAFFIN FROM THE ALIMENTARY TRACT IN THE RAT AND THE PIG.

By HAROLD JOHN CHANNON
and GEORGE ARTHUR COLLINSON.

From the Department of Experimental Pathology and Cancer Research, University of Leeds.

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The researches of a number of workers, which have been undertaken in recent years in an attempt to elucidate the question as to the chemical nature of the unsaponifiable fractions of liver oils, have revealed the fact that such fractions may contain a number of hydrocarbons and alcohols of varying chemical structure. Of the alcohols, mention may be made of the occurrence in certain fish-liver oils of batyl, selachyl and chimyl alcohols [Tsujimoto and Toyama, 1922; Toyama, 1923, 1; 1924, 1, 2; Drummond and Baker, 1929]. The chemical nature of these dihydric alcohols was studied by Tsujimoto and Toyama [1922] and by Toyama [1924, 1] and later by Heilbron and Owens [1928] and Drummond and Baker [1929], who showed that they were the monoglyceryl ethers of octadecyl, oleyl and cetyl alcohols respectively. The presence in fish-liver oils of cetyl and oleyl alcohols has also been reported [Toyama, 1922; 1923, 1], while the latter alcohol has been found also in arctic sperm oil [Tsujimoto, 1925]. In addition to these alcohols, the presence of three hydrocarbons in various liver oils has been demonstrated. The unsaturated hydrocarbon, squalene, occurs in a large number of shark-liver oils [Tsujimoto, 1906; 1917; 1920, 1, 2] and, in traces, in cod-liver oil [Drummond, Channon and Coward, 1925; Drummond and Baker, 1929], and has been shown by Heilbron and his colleagues [Heilbron, Kamm and Owens, 1926] to be a dihydrotriterpene. In mammalian liver, a hydrocarbon of similar chemical nature to squalene occurs [Channon and Marrian, 1926]. A third hydrocarbon is *iso*octadecane which has been obtained from fish-liver oils by Tsujimoto [1917] and Toyama [1923, 2].

The question thus arises as to the source and possible function of these alcohols and hydrocarbons, and in particular whether they are present in these unsaponifiable fractions as a result of their occurrence in the food

ingested, or whether they are synthesised by the organism and play some part in the metabolism of fats or substances of allied nature. With these possibilities in mind, we have been investigating the ability of the mammal to absorb these unsaponifiable substances from the alimentary tract. The study of the absorption of substances other than fats but resembling fats in physical properties began with the researches of Munk, who found that when he administered cetyl palmitate to a patient with a lymph fistula he was unable to demonstrate the presence either of the ester or of the alcohol in the chyle [Munk and Rosenstein, 1891]. Later Connstein [1899] reported that almost all of the 20 g. of lanolin which he administered to a dog was excreted in the faeces, while Henriques and Hansen [1900] on administering a mixture of vaselin and lard to rats found that 95 % of the vaselin was excreted. Again, Bloor [1913] administered a liquid hydrocarbon mixture and also vaselin alone and in emulsions in olive and cocoanut oils, and recovered in the faeces 85–100 % of the hydrocarbons administered. He also gave similar mixtures to animals with thoracic fistulae and prepared the unsaponifiable fraction from the chyle. This was found in every case to be a solid melting about 110° (cholesterol has M.P. 149·5°). These results, therefore, show that although these unsaponifiable substances can be readily emulsified in the small intestine, they remain unabsorbed; and, since fats themselves are readily absorbed, such results provide weighty evidence against the occurrence of particulate absorption. Some of the more recent work, however, seems to suggest that unsaponifiable substances may be absorbed to some extent, at least. Thus, Thomas and Flaschenträger [1923] reported that cetyl alcohol was absorbed to some extent by the dog, and that the rat can also absorb this substance has been shown by Carter and Malcolm [1927] and Channon and Collinson [1928]. In addition, the latter authors carried out experiments with oleyl alcohol and phytol, and absorption of both these alcohols was found to occur in rats. The results of the experiments with phytol, cetyl and oleyl alcohols suggested that their absorption had caused an increase in the amount of unsaponifiable fraction of the liver, although the difficulty of identifying such substances in the small amount of material available prevented their actual isolation. The fact that they are absorbed, however, would possibly explain their appearance in the unsaponifiable fractions of certain liver oils.

The presence of hydrocarbons in the unsaponifiable fractions of liver oils suggested that a study of the question whether hydrocarbons could be absorbed from the small intestine should be undertaken afresh, and it was shown in a previous paper [Channon, 1926] that the unsaturated hydrocarbon, squalene, was absorbed by the rat and appeared in the liver. This finding, coupled with the occurrence of *iso*octadecane in fish-liver oils, made it appear worth while to investigate the question as to whether liquid paraffin was absorbed from the alimentary tract. Our reasons for re-opening this question in the light of much contrary evidence have been that considerable difficulties arise in determining whether absorption of unsaponifiable substances

in small amounts occurs. Hitherto, evidence has been sought in one of two ways. Substances have been administered by mouth and the chyle has been collected and examined for the particular substance, or, alternatively, after administering a known amount of the substance an attempt has been made to determine the amount excreted in the faeces during the experimental period. Apart from the known experimental difficulties of the first method, there is the greater one that the identification of unsaponifiable substances generally is one necessitating a large amount of material and, although the unsaponifiable fraction of the chyle fat may be prepared, the separation of unsaponifiable substances of similar chemical and physical properties is impossible, unless an individual substance constitutes a considerable proportion of the material available. The intake and excretion method suffers from the same difficulty, and in most cases the difference between the weights of the unsaponifiable fraction of the faeces from the control group of animals and that from those receiving the substance has to be taken as the amount of the substance excreted; an uncertain proceeding when it is remembered that faeces normally contain a high and variable content of unsaponifiable substances.

Since the results with squalene showed that this hydrocarbon could be isolated from the animal's liver after absorption, and those with cetyl and oleyl alcohols and phytol also suggested that some part at least of any unsaponifiable material absorbed by an animal would be found in that organ, we decided that a study of the unsaponifiable fractions of the livers of animals which had received liquid paraffin in their diet might provide a more delicate test as to whether the latter were absorbed. As liquid paraffin is a mixture of saturated hydrocarbons, the iodine value of the unsaponifiable fraction of the livers of animals receiving it in their diet should show a fall compared with that of the livers of the animals receiving the control diet only. The unsaponifiable fraction of mammalian livers consists largely of cholesterol, which may constitute up to 85 % of the fraction. Hence it will be necessary to remove the cholesterol quantitatively by means of digitonin, and determine the iodine value of the sterol-free material so obtained, or alternatively to determine the iodine value of the unsaponifiable fraction and to estimate quantitatively the amount of sterol present in it. Calculation of the iodine value of the sterol-free material may then be made, by making allowance for the amount which the cholesterol present contributes to the iodine value. A difficulty with the latter method has hitherto been that cholesterol behaves abnormally with iodine solutions [Smedley-MacLean and Thomas, 1920]. This difficulty has now been overcome by the introduction of the pyridine sulphate dibromide method of Rosenmund and Kuhnhenn [1923] as described by Dam [1925]. This method has been shown by Dam and later by Copping [1928] to give theoretical values with cholesterol, and it possesses the further advantage that it can be applied to very small quantities.

Experimental.

Exps. 1 *and* 2. *Administration of liquid paraffin to rats.*

A control group of rats was fed on the synthetic diet described by Drummond and Coward [1920], while 5 % of liquid paraffin, without previous emulsification, was mixed with the diet of the experimental group. The animals were maintained on these diets for 5 weeks, after which they were kept without food for 24 hours and killed. Their livers were removed and weighed, and the unsaponifiable fractions prepared from them by the method previously described [Channon, 1925]. Aliquot portions of the unsaponifiable fractions were then used for determination of the sterol content by the digitonin method [Windaus, 1910] and of the iodine value [Dam, 1925]. In a second experiment the procedure was exactly repeated. The results so obtained are recorded in Table I.

Table I.

	Animals receiving control diet		Animals receiving control diet plus liquid paraffin	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
No. of animals	5	6	6	6
Wt. of livers, g.	32·0	39·7	41·0	43·5
(*a*) Wt. of unsap. fraction, g.	0·0965	0·1447	0·1860	0·2122
(*b*) Wt. of cholesterol in (*a*), g.	0·0692	0·1200	0·0810	0·0936
(*c*) Wt. of material other than sterol in (*a*) (by difference), g.	0·0273	0·0247	0·1050	0·1186
Iodine value of (*a*) (Dam)	78·6	77·7	47·2	45·1

The results are rendered more lucid by the figures in Table II, in which are recorded the yields of unsaponifiable material per 100 g. of liver and the percentage of cholesterol in the unsaponifiable fraction. With the knowledge of the cholesterol percentage and that cholesterol has an iodine value of 65·8, it is possible to calculate, from these figures and the iodine value of the unsaponifiable matter, the iodine value of that part of the latter which is not sterol. Thus in the control group of Exp. 1, the unsaponifiable matter, which has an iodine value of 78·56, contains 71·69 % of sterol. The iodine value (x) of the non-sterol fraction may thus be calculated as follows:

$$\left(\frac{71{\cdot}69}{100} \times 65{\cdot}8\right) + \frac{28{\cdot}31}{100}\, x = 78{\cdot}56.$$

The results so obtained are also recorded in Table II, together with the mean values of the figures from the two experiments.

From the mean values it is seen that the yield of unsaponifiable matter from the livers of the animals which received liquid paraffin has increased by 40 % over that of the control groups, which suggests that some of the liquid paraffin has been absorbed and has appeared in the liver. This result seems to receive support from the degree of unsaturation of the non-sterol portion of the unsaponifiable fractions, for, whereas the iodine value of the material other than sterol is 118·8 in the control group, it is only 30·8 in the

case of the animals to which liquid paraffin had been administered. Moreover, the percentage of non-sterol material in the unsaponifiable fraction of the livers of the control group is only 22·68, as against 56·17 in the case of the animals which received paraffin.

Table II.

Exp. No.	Animals receiving	(*a*) Unsap. matter per 100 g. liver g.	Cholesterol in (*a*) %	Material other than cholesterol in (*a*) %	Iodine value of (*a*)	Iodine value of the non-sterol fraction of (*a*) (calc.)
1	Control diet	0·3016	71·69	28·31	78·6	110·9
2	,,	0·3645	82·94	17·06	77·7	135·8
1	Control diet plus liquid paraffin	0·4536	43·56	56·44	47·2	32·7
2	,,	0·4877	44·10	55·90	45·1	28·8
Mean values	Control diet	0·3330	77·82	22·68	78·1	118·8
	Control diet plus liquid paraffin	0·4707	43·83	56·17	46·1	30·8

With the other results already reported [Channon, 1926; Channon and Collinson, 1928] figures for the amount of unsaponifiable matter per 100 g. liver and its percentage content of cholesterol have been obtained from determinations on upwards of 200 rats. For this large number of determinations the mean value of the unsaponifiable fraction is of the order of 0·42 g. per 100 g. liver, of which 73 % is sterol precipitable by digitonin, while 27 % is composed of material other than sterol. These figures are mentioned here because, although the percentage of sterol in the unsaponifiable fraction of the control groups in the experiment under discussion is of the same order, 77·82, the yield of unsaponifiable matter per 100 g. of liver, 0·3330 g., is considerably lower than the average value worked out on the large numbers of livers to which reference has just been made. As to the reasons why the yields of unsaponifiable matter per 100 g. of liver in the control groups of these two experiments are considerably below the mean value found for the large number of animals, we have no information, for the diet in this and previous experiments has been the same, although the ages of the rats have varied. Hence no reliance can be placed on conclusions drawn from the figures obtained in these experiments for the yield of unsaponifiable matter per 100 g. of liver only. On the other hand, the significant result of these experiments is that, in the control animals, the material other than sterol in the unsaponifiable fraction constitutes but 22·68 % of the whole and has an iodine value of 118·8 as against 56·17 % and 30·8 for the corresponding figures in the animals which received paraffin. These results seem fairly convincing proof that some liquid paraffin has been absorbed and has found its way into the liver, to cause an increase in the amount of the unsaponifiable fraction and a consequent reduction in the percentage of cholesterol present and the iodine value of the material other than cholesterol. On the other hand, as has been pointed out already, the iodine values of the material

other than sterol have been calculated from the iodine value of the whole unsaponifiable matter by making allowance for the amount of cholesterol present. Since cholesterol gives theoretical results by the iodine method employed [Copping, 1928], this calculation seems to be sound. On the other hand, sterols tend to behave abnormally towards iodine solutions and, since these results seem directly opposed to those of the workers discussed in the opening paragraph of this paper, we have carried out a further experiment on larger animals (pigs) and attempted to isolate the hydrocarbon fraction in some state of purity, to avoid the doubts which calculated results inevitably raise.

Exp. 3. *Administration of liquid paraffin to pigs.*

Two pigs received a diet consisting of a mixture of barley meal 12 parts, white fish meal 1 part, and sharps 6 parts. Before the morning feed, one of the animals received in addition 100 cc. of liquid paraffin administered in the following way. To 100 cc. of gum acacia mucilage (gum 4 parts, water 6 parts) were added 100 cc. of liquid paraffin, and the mixture after very vigorous shaking was thoroughly incorporated in a small portion of the dry diet and given before the morning meal; it was readily consumed by the animal. After 54 days, the animals were killed 36 hours after their last meal. The unsaponifiable fraction of their livers was then prepared and the amount of the sterol present in an aliquot portion estimated by the digitonin method. The results obtained are recorded in Table III.

Table III.

	Animal receiving control diet	Animal receiving control diet plus liquid paraffin
Wt. of liver, g.	1537	1730
„ unsaponifiable fraction, g.	5·6231	8·2420
„ cholesterol, g.	4·231	4·267
„ material other than cholesterol, g.	1·3921	3·975

Note. As to the normal yield of unsaponifiable material from pig liver, we have little information. Two samples bought from shops at different times were worked up for their unsaponifiable content; in one case 935 g. liver yielded 3·47 g., and in the other 659 g. yielded 2·40 g., *i.e.* 3·71 and 3·64 g. per kg. respectively as against the control figure in the present experiment of 3·66 g. per kg.

With a view to the later isolation of the hydrocarbon mixture, it was decided to remove the sterol quantitatively. Accordingly the greater part was obtained by crystallisation of the fractions from light petroleum, boiling below 60°, with which liquid paraffin is miscible. Each fraction was dissolved in 110 cc. of light petroleum and cooled to 0°. The crystalline material obtained by filtration was washed with light petroleum cooled to 0° and the combined mother-liquors were concentrated to 50 cc., and the process was repeated. In this way, there were obtained 3·35 g. and 3·40 g. of sterol from the fractions from the livers of the control animal and the animal which received the liquid paraffin respectively. The remaining sterol was removed

with digitonin. To each of the residues obtained by evaporation of the light petroleum mother-liquors were added 50 cc. of 95 % alcohol, and the mixtures heated to boiling and vigorously shaken. A considerable portion of the material from the liver of the animal which received paraffin failed to dissolve. To each there was then added 3·1 g. of digitonin dissolved in 240 cc. of boiling 90 % alcohol. After gently boiling the mixtures with vigorous shaking, they were allowed to stand overnight. They were then filtered through paper, and the residues, consisting of cholesterol digitonide, excess digitonin and any substances insoluble in 90 % alcohol, were washed with the latter solvent and the washings were united with the mother-liquor, which was then evaporated to small bulk and poured into water. The mixture was then ether-extracted, and the extract washed with water, evaporated to dryness and dried *in vacuo* on the water-bath. The residues on the filters were then thoroughly washed with ether and the ether extract was filtered into a separating funnel. This ether extract was also washed with water and evaporated to dryness, and dried on the water-bath. By this process, each fraction was freed from cholesterol, and divided into two portions, one soluble in about 350 cc. of 90 % alcohol and the other insoluble in that volume of this solvent. The object of the procedure, apart from removing cholesterol, was to concentrate any liquid paraffin present in the insoluble fraction, for the liquid paraffin used in the feeding of the animals was found to have a solubility in 90 % alcohol at room temperature of only 0·18 g./100 cc. Iodine values were then determined by Hübl's method on the four fractions obtained. The weights of the four sterol-free fractions and their iodine values are recorded in Table IV.

Table IV.

	Animal receiving control diet	Animal receiving control diet plus liquid paraffin
Sol. in 90 % alcohol, g.	0·9589	1·3182
Insol. in 90 % alcohol, g.	0·4969	2·5785
Iodine values:		
Sol. in 90 % alcohol	92·8	57·7
Insol. in 90 % alcohol	151·2	25·7

From the figures recorded in this table, the iodine values of the combined sterol-free fractions for each animal may be calculated. In the case of the control animal, the material other than sterol has an iodine value of 112·8 and for the animal which received the paraffin 36·6. These figures may be compared with those obtained in the experiments on rats, the mean values of which were 118·8 and 30·8 respectively, and confirm the results obtained on those animals by a less direct method.

Before describing the attempts made to isolate the hydrocarbons, it will be well to consider the results recorded in Table IV and to discuss the nature of the substances which present-day knowledge suggests may be present. If liquid paraffin be present, it would be expected that the greater

part of the fraction containing it would remain insoluble in the volume of 90 % alcohol used for precipitation of the cholesterol by digitonin. In addition, the fact that liquid paraffin has a slight solubility in 90 % alcohol should cause the amount of the soluble fraction in the case of the animal receiving paraffin to be greater than that in the control. The figures in the table record such a result, for the amount of the insoluble material in the animal receiving paraffin is about five times as great as in the control animal, while there is a corresponding increase of about 0·36 g. in the soluble fraction. Since the paraffin has a solubility of 0·18 %, it might have been thought that this latter difference would be greater considering the volume of solvent used. The reason for it not being so is probably that the separation was carried out in the presence of the flocculent precipitate of cholesterol digitonide which carried down the paraffin with it. It was in the hope of this happening that the procedure adopted was used. A consideration of the iodine values should tell us something of the nature of the substances present. If the soluble material in the case of the animal receiving paraffin contains some hydrocarbon, it should have a proportionately low iodine value. This is so, for the control material has an iodine value of 92·8 as compared with 57·7 for that of the animal receiving paraffin. The differences shown in the insoluble fractions are still more considerable, for the control material has an iodine value of 151·2 as opposed to one of 25·7 for the animal receiving paraffin. It was decided, therefore, to attempt to isolate the saturated hydrocarbons from the fractions insoluble in 90 % alcohol.

Fractions insoluble in 90 % *alcohol.* It was shown in a previous paper [Channon and Marrian, 1926] that there was present in mammalian liver a highly unsaturated hydrocarbon, similar to squalene. This hydrocarbon was not obtained in pure form, but its presence was deduced from the fact that it was possible to isolate it as a bromide and hydrochloride. Since it is very insoluble in any solvent containing water, it should appear in this experiment in the fractions insoluble in 90 % alcohol, and the iodine values of those fractions will be due mainly to the presence of this unsaturated hydrocarbon. It was decided to remove it by taking advantage of the fact that the bromide to which it gives rise is insoluble in ether. Accordingly, the fractions were dissolved in anhydrous ether at 0° and excess of a solution of bromine in ether was added. After standing for a short time, the insoluble bromide from the two fractions was removed by filtration and washed with ether. Each was shown to be the bromide of the unsaturated hydrocarbon, to which reference has just been made, by its bromine content (68 %) and by the fact that it charred at 180° without melting. In this way there were isolated 0·37 g. and 0·5 g. of insoluble bromide from the material from the liver of the control animal and the animal which received paraffin respectively. Since the fraction from the liver of the latter animal had an iodine value of 25·75, 2·45 g. should absorb 0·3975 g. of bromine, while the bromine content of the insoluble bromide isolated is 0·34 g. Hence the bromine content of the

material from which the unsaturated hydrocarbon has been removed is 0·0575 g., or 2·45 % bromine, and hence, prior to bromination, the material present other than the unsaturated hydrocarbon had an iodine value of 4 only. The filtrate and washing from the insoluble bromide were then shaken with sodium thiosulphate solution in order to remove the excess of bromine; and the ether solutions, after washing with water and drying with anhydrous sodium sulphate, were evaporated to dryness. In view of the low bromine content (2·45 %) it was decided to distil the material without debromination. The 2·2 g. were transferred with ether to an 8 cc. flask but, unfortunately, about 20 % of the material was lost during the removal of the ether by evaporation. The material was distilled at 1 mm. pressure. There were obtained two fractions and a residue: fraction (1), 0·3 g. boiling up to 160°, fraction (2), 0·9 g. boiling from 160°–210° and 0·32 g. of residue. The distillates were amber-coloured oils of viscosity similar to that of liquid paraffin.

Fraction (2) analysed as follows:

0·1021 g. gave 0·3202 g. CO_2 and 0·1262 g. H_2O: C, 85·53; H, 13·73.

0·1482 g. absorbed 0·31 cc. of 0·1036 *N* iodine: Iodine value 2·7.

These figures show that fraction (2) approximates sufficiently closely to a saturated hydrocarbon as to render it unnecessary to attempt to purify the remainder further, and the object of this experiment, namely the isolation of the paraffin from the liver, was thus achieved.

Fractions soluble in 90 % *alcohol.* It was considered desirable to attempt to demonstrate whether the increase in the amount of the fraction soluble in 90 % alcohol derived from the liver of the animal which received the liquid paraffin was caused by the presence of that substance. Accordingly, these fractions, 0·864 g. and 1·186 g. from the control and the animal receiving paraffin respectively, were converted into acetyl derivatives by boiling with acetic anhydride. The acetyl derivatives were then saponified by boiling with excess of 0·2 *N* alcoholic potassium hydroxide for 4 hours and the acetyl values so determined. After saponification, the fractions were recovered by ether extraction and brominated in cold dry ether as described in the previous section. After removal of the insoluble bromide of the small amount of the unsaturated hydrocarbon appearing in these fractions, the excess bromine of the mother-liquor was removed by washing with sodium thiosulphate solution. The ether solutions were then dried with anhydrous sodium sulphate and evaporated to dryness, and the bromine content of the materials so obtained determined. The results obtained are given in Table V.

Table V.

	Animal receiving control diet	Animal receiving control diet plus liquid paraffin
Acetyl value	116·6	91·3
Wt. of insol. bromide (g.)	0·14	0·05
Acetyl value corrected for the unsat. hydrocarbon	123·5	92·5
Bromine content after removing the insol. bromide, %	25·68	22·14

It will be seen from the above table that both the acetyl values and the bromine contents of the fractions after bromination are considerably lower in the case of the fraction from the animal which received paraffin, and it seems clear, therefore, that the increased amount of the latter over that of the control animal is due to liquid paraffin appearing in it, which would lower its acetyl value and the percentage of bromine in the brominated product.

Discussion.

The results obtained from the experiment on the pigs in which hydrocarbon has been isolated in nearly pure form leaves no doubt that some part of the paraffin administered has been absorbed and has appeared in the liver. Further, the figures obtained showing the effect of the administration of liquid paraffin on the yields of the unsaponifiable fractions and their iodine values are similar in both the rat experiments and those with the pigs, and if due allowance be made for the biological nature of the experiment, these figures may be used to gain some insight into the question as to how much paraffin has accumulated in the liver of the animals. These figures are set out in Table VI, where the mean values of the two rat experiments are given.

Table VI.

Animals	Diet	Sterol g./100 g.	Non-sterol g./100 g.	Iodine value of non-sterol
Rats (mean values)	Control diet	0·2591	0·0755	118·8
	Control diet plus liquid paraffin	0·2063	0·2643	30·8
Pigs	Control diet	0·2752	0·0905	112·8
	Control diet plus liquid paraffin	0·2467	0·2298	36·6

In the rat experiments, the non-sterol fraction of the livers of the animals which received paraffin is roughly 3·5 times greater than that of those of the control animals, and the iodine value has shown a fall corresponding closely with this increase. In the pig experiment, the increase in the non-sterol fraction is about 2·5 times that of the control, while the iodine value is about one-third as great as the control iodine value, a result in not quite such good accordance as the rat experiments provide.

As to the mechanism by which the absorption of the paraffin has occurred, we have little information. In a previous paper [Channon and Collinson, 1928], the absorption of cholesterol, phytol, cetyl and oleyl alcohols was discussed. The results of Moore and Parker [1901], which extended the earlier observations that bile salt solutions would dissolve fatty acids, by demonstrating that such acids were very much more soluble in bile salt solutions in which lecithin was present, made it worth while to determine the solubilities of these alcohols together with that of squalene and liquid paraffin in bile salt solutions with and without lecithin. Not too great a reliance can be placed on these results on account of the difficulties of such determination and the very low solubilities obtained, but it was found that the alcohols were soluble to an extent in rough proportion to the degree to which they

were absorbed. The solubility of squalene in 5 % bile salt solution at 37° was found to be as low as 0·024 %, while liquid paraffin was found to possess no detectable solubility in this medium. In this connection, the results of Mellanby [1927, 1] are of interest. Mellanby introduced various fat emulsions into the duodenum of a cat and observed fat absorption by inspection of the lacteals in the mesentery, cannulae having been previously inserted into the gall bladder, common bile and pancreatic ducts. He found that emulsified fat to which bile had been added was rapidly absorbed, and absorption caused a secretion of pancreatic juice and an increased flow of bile. On the other hand, neither emulsified fat nor fat previously digested by lipase was absorbed in the absence of bile, and the injections of such mixtures caused no secretion of pancreatic juice and no increase in bile secretion. If there be no available source of lipase other than pancreatic juice, therefore, emulsified fat according to these results may be absorbed without previous hydrolysis. Continuing these experiments, Mellanby [1927, 2] found that in a similar experiment in which an emulsion of paraffin and bile was injected, there was neither absorption of the hydrocarbon nor secretion of pancreatic juice nor an increase in the bile secretion. An experiment which the same author kindly carried out with squalene showed that in contrast to liquid paraffin squalene was absorbed to a small extent, and that the injection caused a large secretion of pancreatic juice and bile. Mellanby's results, both with emulsified fat and squalene, suggest that particulate absorption may occur in spite of much contrary evidence. The results with liquid paraffin recorded in this paper are opposed to those just cited, and in this connection the work of Clark and Clark [1917] may be mentioned. These authors studied the results of the injection of fine emulsions of fat, such as olive oil, oleic acid, egg-yolk and cream, into the tissue of tadpoles' tails. They found that the fat injected was soon surrounded by leucocytes, and lymph vessels grew out to the oil droplet in a time varying from a few hours to two or three days. No reaction, however, was obtained with substances such as mineral oil.

A word may be said here as to the chemical nature of the liquid paraffin used in the experiments. The main fraction boiled at 210° at 1 mm., and since no solid products could be obtained by distillation, and by attempts at crystallisation of the undistilled residues, we were led to the view that the hydrocarbons present in the mixture could not be normal aliphatic substances. This view was confirmed by the figures obtained on combustion, for the mean values showed that the liquid paraffin contained C, 86·6 %; H, 13·3 %. On the other hand, $C_{10}H_{22}$ requires C, 84·50; H, 15·50 and $C_{20}H_{42}$ requires C, 85·1; H, 14·9, and hence a mixture of aliphatic hydrocarbons C_nH_{2n+2} of which liquid paraffin might be composed would have figures intermediate between these such as C, 84·8; H, 15·2, a carbon content definitely lower than that actually found. We consulted Dr W. R. Ormandy on this point and he informed us that, although the popular idea is that medicinal paraffin is composed entirely of aliphatic compounds, strong grounds exist for believing that a large

proportion of it is naphthenes and that some authorities go so far as to consider that the whole of ordinary liquid paraffin is composed of ring compounds. A naphthene $(CH_2)_n$ requires C, 85·7; H, 14·3, while our liquid paraffin corresponds to $(CH_{1·84})_n$. From the combustion figures, therefore, the liquid paraffin appears to consist of a mixture of polycyclic paraffins. This point is important because the nature of the hydrocarbons present in the paraffin may be a factor involved in the absorption[1].

We have no information as to how much liquid paraffin was absorbed by the animals in the experiments described in this paper, but the results of previous workers suggest that it can have been absorbed in small amount only. The fact that some absorption occurs, however, suggests that a study should be made as to whether liquid paraffin administered to a lactating animal can be detected in the milk. This is a question of some practical importance to which we are now giving attention.

SUMMARY.

1. The iodine value of the non-sterol fraction of the unsaponifiable matter of the livers of rats which receive liquid paraffin in their diet was found to be 31 as against 119 for that from the control animals. This result suggests that liquid paraffin is absorbed to some extent.

2. This was confirmed by an experiment on pigs, in which the hydrocarbon was isolated from the liver.

3. The factors concerned in the absorption are discussed.

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LXXVIII. A SECOND THERMOLABILE WATER-SOLUBLE ACCESSORY FACTOR NECESSARY FOR THE NUTRITION OF THE RAT.

By VERA READER.

From the Department of Biochemistry, University Museum, Oxford.

(*Received April 30th, 1929.*)

During the last two years numerous papers have been published confirming the work of Goldberger *et al.* [1926] on the division of the water-soluble vitamin B into at least two components. That further factors may be present has been suggested by Kennedy and Palmer [1928], Hunt [1928] and others. The experiments to be recorded below deal exclusively with the more labile factors concerned. The work was undertaken in order to find the daily requirements of a rat of the antineuritic vitamin in terms of the pigeon day-dose, the method employed in this laboratory for standardising this vitamin. A preliminary account of the work has already appeared [Reader, 1928].

The nomenclature used throughout is that provisionally adopted by the Biochemical Society and defined in detail by Chick and Roscoe [1928]. Briefly, B_1 signifies the antineuritic, and more heat-labile factor; B_2, the more heat-stable factor.

Although control animals on our basal diet + crude marmite grew to adult size at the same rate as those on normal food (corn, bread, lettuce, etc.), repeated attempts to get growth to adult size on basal diet + marmite which has been subjected to alkaline hydrolysis for one hour at 120° + purified extracts of vitamin B_1 have failed. Thus it would seem that at least two factors necessary for rat nutrition had been destroyed by this treatment. Whether or not this second thermolabile substance is identical with that described by Williams and Waterman [1928], as associated with the maintenance of weight and general conditions of adult pigeons, will be discussed later.

It is proposed to call this second thermolabile rat factor, provisionally, vitamin B_3.

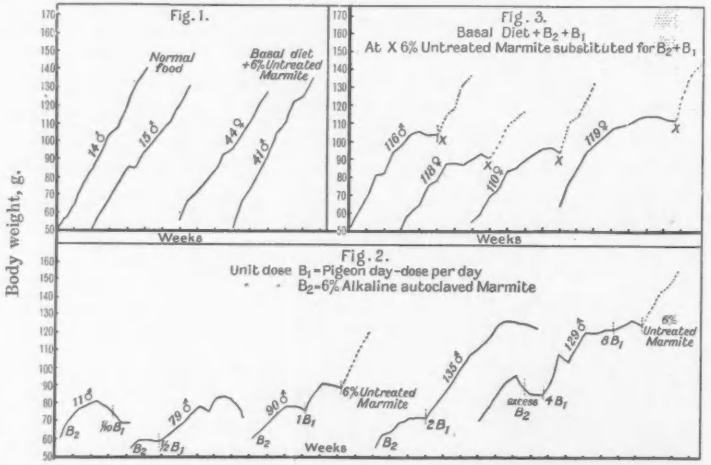
Experimental.

Albino rats, bred in this department from the Wistar strain supplied by Glaxo Limited, were used throughout. In all routine experiments animals of 50–60 g. were used for these tests. However, in order to compare these experiments with those reported by the Lister Institute, one set of animals of about 40 g. was put on to the experimental diet (see Fig. 5).

The basal diet used was:

Glaxo caseinogen (free from vitamins A, D, B_1 and B_2) ...	20 %
Rice starch	70
Agar-agar	2
McCollum's salt mixture	5
Cod-liver oil	3
Water *ad lib.*	

This diet was freshly prepared daily, and was tested to show that, when supplemented with 6 % marmite as source of B_1 and B_2, it gave growth equal to that obtained with normal food, including corn, "Hovis" bread, lettuce, cod-liver oil, and occasionally milk. The curves in Fig. 1 are selected from groups of 20 animals and are typical of the group. The daily food intake gradually rose from 10 to 15 g. as the body-weight increased from 60 to 120 g., so that in all each animal was having daily B_1 and B_2 equivalent to 0·6–1·0 g. marmite.



In all further experiments the thermostable factor or factors (B_2) were supplied by adding 6 % autoclaved marmite solution (120° for one hour at p_H 9·0) to the basal diet. Curative tests on pigeons showed that this product did not now contain any B_1, the antineuritic factor. That it did contain one factor necessary for the rat (presumably B_2) was shown by the method of Chick and Roscoe [1928] for the assay of B_2. Also that 6 % was sufficient for the daily requirements was demonstrated by doubling this quantity after growth ceased in one group of rats (*e.g.* rat 129, Fig. 2) and noting that no further growth occurred.

Two types of experiments were designed. In (A) the rats were fed on the basal diet + B_2 till constant weight was obtained for three consecutive

weighings (three-day intervals). Then varying doses of several B_1 concentrates were administered daily, in aqueous solution adjusted to p_H 6·5. The doses were given by the mouth from a small pipette. The concentrates used were prepared by Mr H. W. Kinnersley[1]. Unless otherwise stated they were the 50 % alcohol extracts from the charcoal. Daily doses were varied from 1/10 to 8 pigeon day-doses. Typical results from a large number of experiments are shown in Fig. 2. From these it may be seen that even eight pigeon day-doses per day do not give growth at a normal rate to adult size.

In (B) (Fig. 3) there was no preliminary lag period to constant weight as the animals were fed from the start on basal diet + B_1 + B_2. It will be seen from Fig. 3 that in every case growth ceased or proceeded at a very subnormal rate after 4 or 5 weeks. Immediately untreated marmite was substituted as source of B_1 and B_2 growth was resumed to maximum weight.

Thus it would seem clear from Figs. 1, 2 and 3 that a second factor, other than B_1, had been destroyed by the alkaline hydrolysis of the marmite.

0·1 N HCl extracts of B_1.

Experiments of type (A) above were arranged with B_1 concentrates prepared by extraction with 0·1 *N* HCl from charcoal in place of the 50 % alcohol extracts used above. Results were similar to those in Fig. 2, but slightly more marked, so presumably these extracts were more free from the second thermolabile factor than the 50 % alcohol extracts (see Fig. 4).

Use of younger animals.

As four weeks' feeding was needed in many of the above experiments before the absence of the second thermolabile substance became evident, it was now decided to carry out a set of experiments using younger animals (40 g. instead of 60 g.), in order to compare these results with those reported from the Lister Institute. As was expected, these animals were more sensitive to the lack of B_3; in fact, some of them died within a week of the time they began to drop in weight. However, if carefully watched and fed with untreated marmite they completely recovered (Fig. 5).

Properties of second thermolabile substance.

(*a*) It is generally agreed that alkaline hydrolysis for one hour at p_H 9 at 120° destroys the polyneuritis-curative properties of an aqueous suspension of marmite, yeast, etc. If this heating is carried out at a more acid reaction (p_H 6)[2] destruction of vitamin B_1 does not occur, but from the

[1] *Note by H. W. Kinnersley.* The concentrates used in the above experiments were the 0·1 *N* HCl or 50 % alcohol extracts from charcoal. In each case, after removal of metallic ions by H_2S, they were fractionated into 99 % alcohol [see Kinnersley and Peters, 1928, pp. 425, 426].

[2] I am indebted to Prof. R. A. Peters for testing these solutions. He has found that they retain at least 50 % of their curative properties when autoclaved under these conditions. The matter is still under investigation.

curves in Fig. 6 (rats 167 and 104) it would appear that there has been more than 50 % destruction of B_3. As would be expected, upon the view that B_1 is not destroyed, the early part of the curves is similar to those obtained with B_2 + the less impure extracts of B_1. Additional indirect proof was obtained by adding B_1 at the point where growth first ceased and noting that no increase in weight was observed (see Fig. 6, rat 104).

(*b*) From the above experiments it seemed clear that B_3 is more thermolabile than B_1. Further proof of this was obtained by boiling marmite in an open vessel on a water-bath for 2 hours (the actual temperature of the marmite was 95°–97°). More than 50 % destruction occurred. 12 % marmite in the diet did not give growth equal to that previously obtained with 6 % (Fig. 6, rat 152).

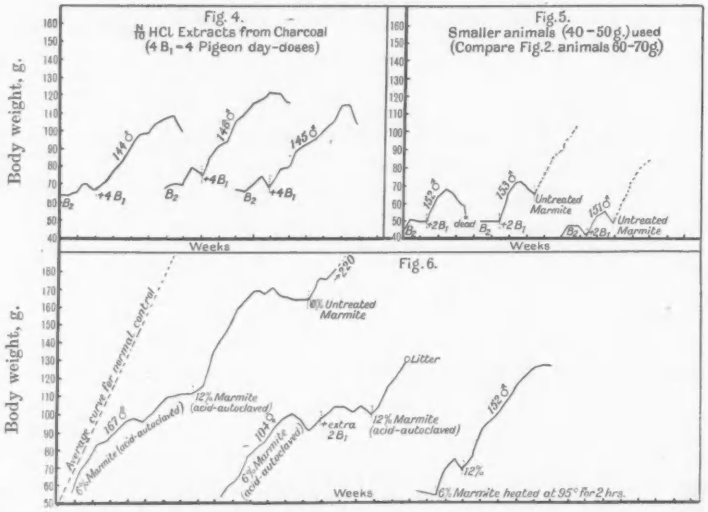


Fig. 6. Partial destruction of second thermolabile factor
(*a*) by acid hydrolysis at 120° for 1 hour;
(*b*) by heating at 95° for 2 hours.

(*c*) *Solubility in ether.* Six hours' extraction with ether in a Hurtley extractor removes most of the B_3. However, if the ether is now distilled off, the substance cannot be redissolved in fresh ether. Thus it does not seem to be a true solubility of the substance itself, but rather dependent on adsorption upon associated substances. Further work upon this point is being carried out in the hope of getting a supply of B_3 free from B_1.

Discussion.

From the above experiments it would seem established that when marmite is subjected to alkaline hydrolysis at 120° for one hour at least two factors necessary for rat nutrition are inactivated. One of these is the antineuritic vitamin B_1. It is suggested that the other is a second thermolabile factor B_3. Evidently B_1 concentrates can be prepared free from this second factor by the extended method of Kinnersley and Peters [1928], and it is due to the advantage of having these more pure concentrates at my disposal that I have been able to demonstrate the absence of the second thermolabile factor from diets complete in other respects. However, so far a sample of B_3 has not been prepared free from B_1: therefore it cannot be definitely proved that the lack of growth is not due to the racemisation of some amino-acid or similar substance. In fact this view is supported by the fact that acid hydrolysis at 95° and at 120° apparently cause a certain definite loss of efficiency (> 50 %) whether the heating be for one hour or two: but absolute destruction of both B_1 and B_3 occurs on alkaline hydrolysis at 120° for one hour.

Williams and Waterman [1928] have recently reported the presence of a very thermolabile factor in yeast necessary for the maintenance of the weight and general condition of pigeons. However, they were unable to show its necessity for the rat. Whether they observed complete destruction of their factor by heating is not stated. As their preparation of B_1, made by the fuller's earth method, was not heated in the process, the B_3 reported in my experiments was probably still present associated with their B_1; hence they would not get a cessation of growth due to lack of this factor. Moreover, certain preliminary experiments carried out in this department (Peters, private communication), indicate that the second rat factor does not restore the weight of pigeons; thus the evidence seems to be in favour of two further factors—one, reported by Williams and Waterman, for pigeons: the other, reported in this paper, for rats.

At first sight these results seem to be contradictory to the results of Chick and Roscoe [1928]. In their work on the assay of B_2, they used B_1 concentrates prepared by the method of Kinnersley and Peters [1927] but not purified by alcohol fractionation, so it is possible that traces of B_3 were still present. However, it must be emphasised that in their actually recorded experiments they assessed their results from the growth obtained in the first 2 weeks after addition of B_2 to diets apparently complete in other respects. This procedure employs the justifiable assumption that if a diet is deficient in more than one factor, an immediate response is obtained by addition of any one of these. If, however, they had continued their experiments for 4 or 5 weeks, the picture presented might have been a different one.

SUMMARY.

Evidence is presented for the destruction of at least two factors, necessary for the nutrition of the rat, by alkaline hydrolysis of yeast extract at p_H 9 at 120° for one hour.

The conclusion is therefore drawn that a second thermolabile factor (B_3) is present in yeast extract.

My thanks are due to Professor R. A. Peters for his interest and criticism throughout the work, to Mr Kinnersley for the supply of B_1 concentrates, and to the Medical Research Council for a grant.

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LXXIX. SOME EVIDENCE OF THE EXISTENCE OF A FURTHER FACTOR NECESSARY FOR GROWTH OF THE RAT.

By KATHARINE HOPE COWARD, KATHLEEN MARY KEY and BARBARA GWYNNETH EMILY MORGAN.

From the Pharmacological Laboratories, Pharmaceutical Society, 17, Bloomsbury Square, London, W.C. 1.

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The experiments described in this paper were designed to ascertain how far the vitamin A-free diet in use in this laboratory could, when supplemented by cod-liver oil, support normal growth. In the early work of this department (1927) there had been nothing in the behaviour of the rats used in assaying cod-liver oil to suggest any deficiency in the diet other than vitamin A, for the addition of this substance in a dose of 0·01 g. daily caused resumption of rapid growth in the test rats for a period of 9 weeks, after which the test was discontinued (Fig. 1). It was several months later that a

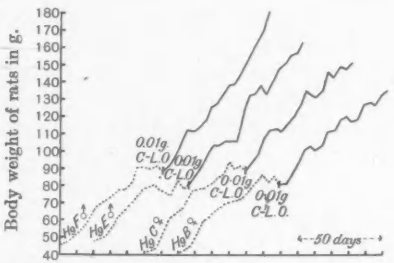

Fig. 1. A test for vitamin A in cod-liver oil. Rats given "vitamin-free casein" (Glaxo) throughout the experiment. Evidence that some rats, at least, can grow well when this casein is the source of protein.

deficiency other than vitamin A was suspected in the vitamin A-free diet and possibly also, though to a smaller extent, in the diet of the stock colony.

The history of the attempt to breed a colony of rats on some diet that should be uniform throughout the year may form the subject of a separate communication. The result of the first stage of it may be summarised as a progressive deterioration of the litters obtained from the imported mature animals, both in respect of their rate of growth when allowed to grow to maturity for breeding and later in their failure to rear the young they had borne. A progressive decrease in the periods of time required for the young

rats to become steady in weight when given the vitamin A-free diet, and a lower weight at which they ceased growth was also noticeable. The whole of these results could, so far, be attributed to a low vitamin A content in the diet of the stock rats with the consequent low reserves of this factor in the rats used for vitamin A assays. It became evident, however, that the responses to doses of cod-liver oil given by these particular rats were definitely less than the responses obtained in rats of different history. Moreover, increasing the dose of vitamin A gradually to a very high dose (0·2 g. daily) of a sample of cod-liver oil (which in other rats caused resumption of good growth in a dose of 0·01 g.) failed to bring about any increased rate of growth. It was evident that the diet was lacking in some factor other than vitamin A.

Coward, in 1926, discussed with Prof. Palmer his work on the growth-promoting powers of different samples of caseinogen prepared in different ways, and it appeared at once possible that the rats in the experiments described in this paper were suffering from a deficiency such as Palmer had noted in his work. The first substance examined for the unknown factor was therefore a fresh kind of caseinogen. The British Drug Houses "light-white casein" was substituted for the Glaxo "vitamin-free casein" of the diet previously used. The response was immediate and dramatic (Fig. 2). The rats grew rapidly and gave no further evidence of any deficiency in their diet. Although Palmer's work has finally indicated that his rats were suffering from a shortage of the vitamin B_2 [Palmer and Kennedy, 1927, 1, 2; 1928, 1, 2], yet further work in this laboratory has shown that the "light-white casein" (B.D.H.) contains neither B_1 nor B_2, but some growth-promoting property which is independent of both of these. The same factor has been found in various natural foodstuffs. It is at least partially destroyed by heat; it is extracted slightly from the casein by hot alcohol and by hot ether after cold alcohol. It seems to be more easily extracted by hot ether from wheat embryo. It is not vitamin E.

Technique.

Rats from various colonies were used in the early tests carried out in this department. That they varied greatly in their reserves of vitamin A was evident from their varied behaviour when given the vitamin A-free diet of this laboratory. Litters were kept intact and members of one litter were, in general, exhausted of their reserves of vitamin A within a very few days of each other. There were much greater differences in vitamin A reserves between different litters than between different members of any one litter.

The vitamin A-free diet consisted of:

Dextrinised rice starch	71 %
"Vitamin-free casein" (Glaxo)	15
Agar-agar	2
Dried yeast	8
Salt mixture (Steenbock's)	4

The mixture was irradiated in very thin layers (200 g. over an area of 4 sq. ft.) in zinc trays for 30 minutes at a distance of 2 ft. from a quartz mercury vapour lamp, running at 2·5 amp., 130 v. It was stirred and re-spread after the first 15 minutes of irradiation. This method has been shown by Steenbock and Coward [1927] to generate ample supplies of vitamin D; and that it is also adequate with the materials available in this laboratory has been shown from time to time by experiment. Rats which had ceased growth on this diet have been given large daily doses of irradiated ergosterol and have not resumed growth.

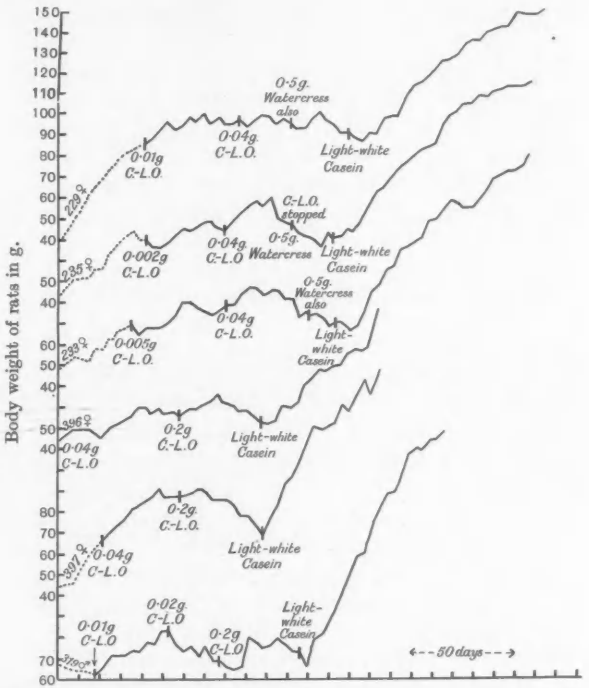

Fig. 2. A test for vitamin A. Rats given "vitamin-free casein" (Glaxo) at first. Growth ceased in spite of the doses of cod-liver oil being greatly increased and in spite of the addition of watercress. Growth was only resumed when "light-white casein" (B.D.H.) was substituted for the "vitamin-free casein."

The rice starch was partially dextrinised by mixing it with enough water to make it firm, and then heating it in shallow trays in an ordinary gas cooking stove for 6 hours, which renders it crisp and easily ground. The dextrinisation of rice starch does not seem to be necessary for making it more easily digested, as with the corn starch used in American laboratories. Dextrinisation is necessary, however, to get the starch into a less fine state of division, for in

its original state the rats collect a great deal of it in their fur and the whole experiment is less clean than when dextrinised and ground starch is used.

The "vitamin-free casein" supplied by the Glaxo Company was guaranteed substantially free from vitamin A by tests which were until recently supervised by the Accessory Food Factors Committee of the Medical Research Council and Lister Institute.

Agar-agar was incorporated in the diet as a preventive of constipation, although, in view of the large percentage of dried yeast also used, it is very doubtful whether it was necessary. Sometimes the powdered form of agar-agar was used, sometimes the shredded. When the latter was used it was eaten as readily as the rest of the food. It was not washed or otherwise purified before use.

The dried yeast used in this diet was brewer's yeast. Every sample purchased for use in this laboratory is tested for its vitamin B complex and there is ample evidence that the inclusion of 8 % of it in the vitamin A-free diet is abundant for the vitamin B needs of the rat at all stages of growth. The evidence rests on the ability of rats, from some colonies at least, to grow rapidly (bucks to more than 200 g., does to about 180 g.) when this is the only source of the vitamin B complex in their diet.

The rats were kept on screens to prevent coprophagy only when they were being used for a vitamin B test. Otherwise they were kept on shavings or sawdust.

The evidence for the existence of a growth-promoting factor other than the vitamins A, B_1 and B_2, D and E in "light-white casein" (B.D.H.).

The general scheme of the experiments which led to the detection of the absence of this factor from the "vitamin-free casein" of the vitamin A-free diet and of its presence in the particular sample of untreated (*i.e.* "light-white," B.D.H.) casein examined later, was as follows. In addition to those experiments with rats which had failed to give the expected response to doses of cod-liver oil, others were performed with rats which had given a certain amount of response under the same condition. In the latter experiments the vitamin A tests of the samples of cod-liver oil were carried on for much longer periods than that of 5–6 weeks usually considered necessary for determining the potency of the oil. In general, the rats ceased to grow and would not resume growth on being given larger and larger doses of cod-liver oil, or if growth were resumed it was only for a short time. Watercress, as an additional and different source of vitamin A, was then tried on some of these rats. A dose of 0·5 g. of it caused no resumption of growth, although an independent test [Coward and Eggleton, 1928] had shown that in the normal way of testing, when the rats were only about 10 weeks old, a dose of 0·1 g. had been enough to restore growth. Unlimited supplies of watercress were then given to the rats which had failed to grow on 0·5 g. of it, and although growth was resumed for a time, it was only temporary. Then "light-white casein"

(B.D.H.) was substituted for the "vitamin-free casein" (Glaxo) and rapid and prolonged growth was resumed. At this stage of the investigation, a discussion on vitamin A assay in cod-liver oil with Dr J. W. Trevan revealed the fact that he had encountered the same difficulty in raising rats to maturity on a vitamin A-free diet plus cod-liver oil. He had found that only by giving such rats large quantities of fresh grass clippings could they be made to resume growth.

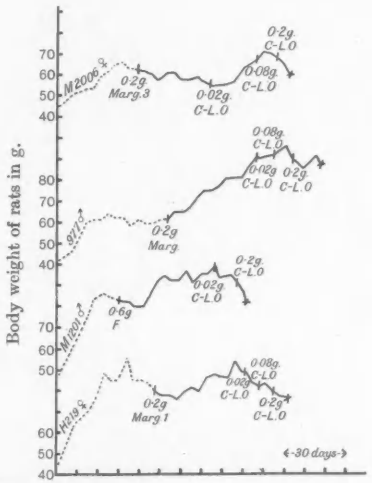

Fig. 3. Growth curves for comparison with those in following figures. If the lately recognised factor necessary for growth is withheld, death ensues. These are typical of some 70 such curves obtained during this work.

Other sources of the supplementary growth factor.

It has been for many months now a matter of routine in this laboratory to keep rats which have already been used for a vitamin A test for further work on the unknown factor. If they have not reached maturity at the end of the recognised test period, they are deprived of their vitamin A-containing substance until they begin to fall in weight. They are then given 0·02 g. daily of a good sample of cod-liver oil. If they do not resume growth within 2–3 weeks the dose is doubled and in time increased to 0·2 g. The great increase in dosage of cod-liver oil has seldom resulted in any increased growth.

It has therefore been possible to test several other food substances and preparations on a reasonable number of rats, for only the animals which had received very large doses of vitamin A from the beginning of the test period, and so had grown rapidly, or rats which had been used for assaying the vitamin A content of milk, failed to respond to the later treatment adopted for exhausting their reserves of the unknown factor.

The distribution of the factor, so far studied, may be summarised thus:

Table I.

Substance	Dose tested	No. of rats used	Results
1. "Light-white casein" (B.D.H.) untreated, 4 different samples	15 % of diet	40	Rapid growth in all but 3 rats, slow growth in these
	10 % of diet	4	Good growth but less rapid than when 15 % was given
	5 % of diet	4	No growth
2. "Crude casein" (B.D.H.)	15 % of diet	4	1 died. 3 maintained weight for 75, 49, 30 days respectively*
3. Dairy butter	0·5 g. daily	3	Maintained weight for 34 days
4. Fresh milk (London supply)	10 cc. daily	4	Rapid growth in all
5. Milk boiled for 15 mins.	10 cc. daily (=15 cc. fresh)	4	Moderate growth in all
6. Lettuce—fresh	1 g. daily	7	Maintained weight in 5 for 7–30 days. Fair growth in 2
	Ad lib., often 40–50 g. per rat per day	7, the same animals that had 1 g.	Good growth
7. Etiolated wheat shoots, 10 days old (grown under laboratory conditions)	15 g. daily	3	Slight growth
8. Dried grass clippings	5 % of diet	3	Rapid growth in 2 rats. Slow growth in 1 rat increased by raising amount to 10 %
9. Fresh grass clippings	About 10 g. daily	2	Rapid growth
10. Extra dried yeast	10 % of diet	14	No influence*
11. Marmite	0·5 g. daily	13	Slight temporary increase in weight in 5. Maintained weight in others†
12. Autoclaved marmite	1·0 g. daily	3	No influence†
13. Watercress	*Ad. lib.*, about 10 g. daily	3	Slight increase†
14. Ox muscle	1 g. daily	4	Good growth
	Ad. lib, 15–20 g. daily	4	Very rapid growth
Ox liver	1 g. daily	4	Very rapid growth
15. Wheat embryo	5 %	3	Rapid growth in 2. Weight maintained in 1
	10 %	3	Rapid growth
16. Wheat germ oil, sample 0	Up to 0·08 g.	6	No influence‡
	Up to 0·16 g.	4	1 maintained weight. 2 fair growth. 1 died
Wheat germ oil, sample 1	Up to 0·02 g.	3	No influence
	Up to 0·12 g.	3	No influence in 2. Fair growth in 1
Wheat germ oil, sample 2	0·12 g. daily	4	Fair growth in 3. Maintained weight in 1

* One of these rats was finally given "light-white casein" to which it responded with rapid growth.

† Two of these rats were finally given "light-white casein" to which they responded with rapid growth.

‡ One of these rats was finally given 10 % wheat germ to which it responded with rapid growth.

Similar tests were made with (*a*) liquid paraffin (0·06 g. daily for 21 days) and (*b*) 3 % extra agar-agar in the diet to see whether the active substances had merely been exerting an influence on peristalsis. Each substance was without effect on the growth of the test animals.

The palatability of the two diets containing the "Glaxo casein" and the "light-white casein" respectively.

A simple experiment was carried out to see whether possibly greater palatability of the "light-white casein" diet could account for the observed results. Six young rats weighing about 60 g. were divided into two groups of 3 each. One group was given the vitamin A-free diet containing the "vitamin-free casein" (Glaxo) and the other the diet containing "light-white casein." All the rats were given 0·02 g. of a good sample of cod-liver oil daily to make the same rate of growth possible in all of them. Records of food consumed each day were taken at 9 o'clock in the morning in order to get figures as accurate as possible. After 2 days the diets of the two groups were interchanged. This was repeated 5 times in all and then each group was kept to one diet for a period of 5 days, and the diets were again interchanged for a further period of 5 days. The amounts consumed in g. are given in Table II.

Table II.

Rat	M 347		348		349		350		351		352	
Casein in diet	G.	L.-W.	G.	L.-W.	G.	L.-W.	G.	L.-W.	G.	L.-W.	G.	L.-W.
27. iii. 28	7	—	5	—	7	—	—	7	—	3	—	3
28	6	—	7	—	5	—	—	7	—	5	—	5
29	—	7	—	5	—	7	9	—	7	—	8	—
30	—	7	—	5	—	7	9	—	7	—	8	—
31 (2 days)	13	—	14	—	17	—	—	19	—	16	—	17
2. iv. 28	—	5	—	2	—	8	10	—	8	—	9	—
3	—	5	—	2	—	7	10	—	8	—	8	—
4	5	—	6	—	9	—	—	9	—	9	—	8
5	6	—	6	—	9	—	—	9	—	8	—	9
6	—	5	—	7	—	8	11	—	9	—	11	—
7 (2 days)	—	10	—	10	—	12	14	—	14	—	14	—
9	—	2	—	4	—	(Spoilt) 12?	4	—	4	—	4	—
10 (2 days)	—	15	—	20	—	20	20	—	21	—	19	—
12	6	—	9	—	8	—	—	10	—	9	—	9
13	7	—	9	—	11	—	—	12	—	10	—	10
14 (2 days)	12	—	17	—	22	—	—	28	—	22	—	27
16	8	—	10	—	11	—	—	12	—	10	—	11
17	—	8	—	10	—	14	14	—	12	—	11	—
Total for 11 days	70	64	83	65	99	95	101	113	90	92	92	99

An examination of this table shows that

(*a*) there is no significant difference between the total amounts of the two diets eaten by any one rat during the experiment, and that

(*b*) there are practically no instances of a greater consumption of the "light-white casein" diet on the first day of receiving it than of the diet containing the "Glaxo casein." The evidence shows rather the reverse of this.

Hence the possibility of these results being due to the greater palatability of the "light-white casein" diet must be discarded.

Effect of heat on the growth-promoting power of "light-white casein."

I. "Light-white casein" was heated in very thin layers in open trays in an electric oven (300 g. over 8 sq. ft.) for 24 hours at 105°. It was raked over twice during this time. It was then given to rats (5) as 15 % of their diet. They grew as well as the rats receiving 15 % of untreated "light-white casein" and it was evident that there had been no appreciable diminution in the potency of the casein.

II. "Light-white casein" was heated as above for 7 days, and then tested in the same way. Again the response was good but definitely less than before.

III. Milk which had been simmered for 15 minutes in an open pan lost a great part, though not all, of its activity.

Attempts to extract the growth-promoting substance.

I. *From "light-white casein."*

Extracts of the "light-white casein" made with (*a*) cold 97 % alcohol (4 times), (*b*) cold ether (4 times) after cold alcohol (once) and evaporation *in vacuo* both failed to cause any resumption of growth, while the casein so extracted showed no impairment of its growth-promoting powers. Similar results were obtained with a 2 % acetic acid extract, neutralised with potassium hydroxide and evaporated on a water-bath, and with the casein thus extracted.

Rather more active extracts were obtained by the use of (*a*) hot alcohol (4 extractions of 6 hours each) and of (*b*) hot ether after drying overnight (4 extractions of 6 hours each). The residual casein showed somewhat impaired activity. The extracts described above were all tested in doses equivalent to the amount of casein eaten per rat per day. Slight activity was also shown by an ether extract of a solution of casein in sodium carbonate, which was tested in a dose equivalent to 5 times the daily intake of casein. The growth-promoting factor is therefore not easily removed from the casein by the usual fat solvents.

II. *From wheat embryo.*

(*a*) *Hot* 90 % *alcohol.* Extracting wheat embryo with 90 % alcohol 4 times by boiling for 6 hours each time removes the active factor almost completely and gives an active extract. The yield of this extract was about 30 % (95 g. and 87 g. respectively from two portions of 300 g. each). When given to two rats in doses of 0·05 g. daily (equivalent to 0·15 g. wheat embryo), there was no response. When given in larger doses (1 g. and later 2 g.), one rat resumed rapid growth immediately and another after a period of about 30 days. Another, having remained steady on about 1 g. per day for 80 days,

resumed rapid growth when the amount was doubled. Three rats given the extracted wheat embryo grew very slowly for about 60 days but resumed rapid growth immediately non-extracted wheat embryo was substituted for the extracted product (Fig. 4).

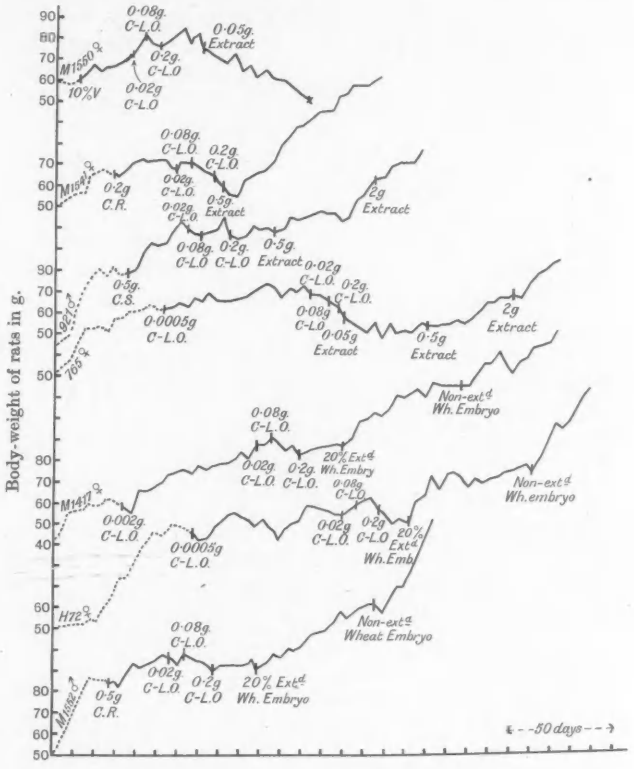

Fig. 4. A 90 % alcohol extract of wheat embryo. Definite activity of the extract and impairment of the activity of the wheat embryo.

(*b*) *Hot ether.* Wheat embryo was dried overnight in an oven at 105° and extracted 4 times with boiling ether. The ether was distilled off at atmospheric pressure. The extract was decidedly active in a dose of 0·16 g. and more so in one of 0·24 g. The extracted wheat embryo was definitely less active than the non-extracted product (Fig. 5).

Evidence from vitamin B testing of the existence of a growth-promoting factor in "light-white casein" (B.D.H.).

In view of the slight temporary response to extra doses of vitamin B in the form of marmite, which was occasionally experienced in the experiments

already described, "light-white casein" (B.D.H.) was tested first for the whole B complex, then for B_1 and B_2 independently. It was found to contain neither of these but to contain a third factor essential for growth and independent of B_1 and B_2.

Technique. The diet on which rats have been prepared for vitamin B testing in this laboratory (until a few months ago) was composed of:

Dextrinised rice starch	79 %
"Vitamin-free casein" (Glaxo)	15
Agar-agar	2
Salt mixture (Steenbock's)	4

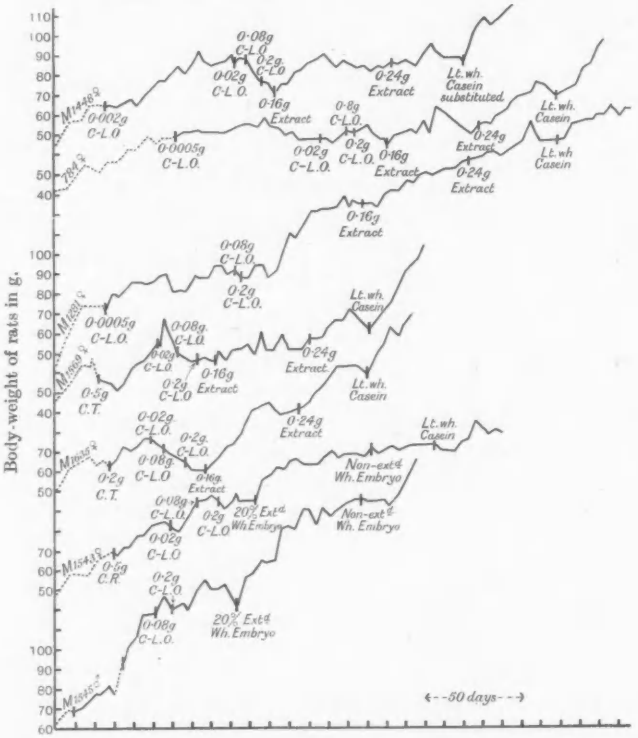

Fig. 5. A hot ether extract of wheat embryo. Some activity of the extract and slight impairment of the activity of the wheat embryo.

Each rat was given separately 5–6 drops of a good sample of cod-liver oil twice a week. In 10–14 days on this diet, the rats became steady in weight or declined.

To test "light-white casein" for the full vitamin B complex, it was substituted for the "Glaxo casein" (15 %) in the diet of 5 rats prepared as just

described. None of the animals showed any tendency to resume growth. They all declined in weight steadily and died in 10–20 days. The "light-white casein" was therefore considered to be devoid of the vitamin B complex.

To test "light-white casein" for B_1 and B_2 independently of each other, 8 rats were prepared on the diet described above. The scheme of the experiment was to determine whether "light-white casein" could produce growth if the rats were given in addition (*a*) excess of B_1, (*b*) excess of B_2, (*c*) excess of both B_1 and B_2.

The B_1 preparation used in this experiment was very kindly given to the authors by Prof. J. C. Drummond, who had found that the dose required to make a rat grow in the presence of ample supplies of B_2 was about four times the amount required to cure a pigeon of polyneuritis. The pigeon dose of this particular preparation was 0·1 cc. (confirmed in our own laboratories) and in order to ensure excessive doses of B_1, 0·8 cc. was used for each rat.

The B_2 preparation was also supplied by Prof. Drummond. It was made by autoclaving marmite (decidedly alkaline) at 125° at 15 lbs. pressure for 3 hours. It was then made very slightly acid with HCl and the volume made up so that 1 cc. was equivalent to 0·4 g. of the original marmite.

The 8 rats were divided into four groups and given the following doses:

Group I.	0·8 cc. B_1 preparation per rat per day. 1·0 cc. B_2 ,, ,,
Group II.	0·8 cc. B_1 preparation per rat per day. "Light-white casein" in place of "vitamin-free casein."
Group III.	1·0 cc. B_2 preparation. "Light-white casein" in place of "vitamin-free casein."
Group IV.	0·8 cc. B_1 preparation. 1·0 cc. B_2 ,, "Light-white casein" in place of "vitamin-free casein."

After 16 days, the dose of B_2 was doubled, as several workers have declared lately that the vitamin B potency of marmite is somewhat less than formerly.

The result is seen in Fig. 6. Even the high doses of B_1 and B_2 have failed to produce normal growth in the animals of Group I. The animals of Group II have grown considerably less than those of Group I, thus showing that "light-white casein" supplies no appreciable quantity of B_2. The animals of Group III died sooner than did those of Group II, thus showing that "light-white casein" does not contain B_1. The animals of Group IV resumed rapid and prolonged growth till the experiment was terminated at the end of 8 weeks. It is therefore evident that "light-white casein" contains some factor necessary for growth which is not one of the vitamins A, B_1, B_2 or D.

Two questions then presented themselves.

(*a*) Why was any growth obtained either by ourselves or by other workers when rats, prepared as these, were given preparations of B_1 and B_2?

(*b*) Is the factor here demonstrated the same one as was demonstrated by the experiments previously described in this paper?

The first question can apparently be answered (*a*) by assuming the rat's ability to store an unknown factor such as is contained in the "light-white casein" or (*b*) by assuming the presence of this growth-promoting factor in the vitamin A-free diet, but in a much smaller degree than in the "light-white casein."

The second question can at present only be answered by repeating the tests on the substances used in the first set of experiments (vitamin A technique), using now the technique described immediately above; *i.e.* testing each substance by the vitamin B technique in the presence of excess doses of B_1 and B_2.

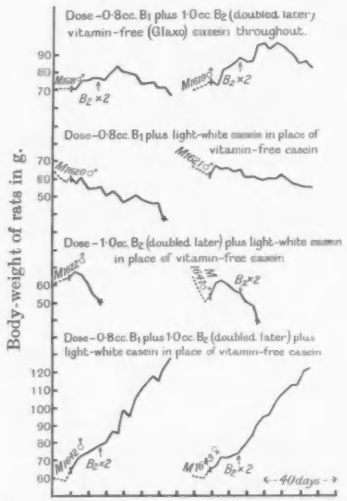
Dose–0·8cc. B1 plus 1·0cc. B2 (doubled later) vitamin-free (Glaxo) casein throughout.
Dose–0·8cc. B1 plus light-white casein in place of vitamin-free casein
Dose–1·0cc. B2 (doubled later) plus light-white casein in place of vitamin-free casein
Dose–0·8cc. B1 plus 1·0cc. B2 (doubled later) plus light-white casein in place of vitamin-free casein
Body-weight of rats in g.
B2 ×2
40 days

Fig. 6. To demonstrate the presence of a growth-promoting factor in "light-white casein" which is not in the "vitamin-free casein" and which is neither vitamin B_1 nor B_2.

Two experiments on this latter point have been carried out.

In the first experiment, 4 rats were prepared on the "B" diet containing the "vitamin-free casein" (Glaxo). After 11 days, they were all given daily doses of 2 cc. of the same preparation of B_2 as in the last experiment and 0·1 cc. of a B_1 preparation very kindly given to the authors by Dr H. Chick, who had found this dose of her preparation ample for the growth needs of the rat. One of the 4 rats continued to receive the "Glaxo casein"; it grew slowly and 21 days after the beginning of the test period had gained 17 g. in weight. Two rats were given "crude casein" (B.D.H.) in place of the "vitamin-free casein" (Glaxo) and at the end of the experiment (21 days) had gained 2 and 7 g. respectively. The fourth rat was given "light-white

casein" (B.D.H.) and had gained at the end of the experiment (21 days) 52 g. in weight. This would appear to indicate that the "crude casein" (B.D.H.) was as nearly free from the unknown factor as the "Glaxo casein."

In the second experiment a comparison was made between (*a*) the "vitamin-free casein" (Glaxo), (*b*) the "light-white casein" extracted once with alcohol and four times with ether in the cold, and (*c*) untreated "light-white casein." The same doses of B_1 and B_2 as in the last experiment were given to all the rats. At the end of 31 days, the one rat given "vitamin-free casein" had gained 17 g. in weight, the two given the extracted "light-white casein" had gained 37 and 32 g. respectively, while the one given untreated "light-white casein" had gained 49 g. It was evident that some of the active factor, but by no means all of it, had been extracted from the casein by the method adopted.

These two experiments have given the same results for the "crude casein" and for the extracted "light-white casein" which were given in the previous method of testing.

It would appear, then, that this offers an alternative method of testing for the unknown factor. Actually, by the time the rats are ready for the test of the unknown substance, they are receiving the same diet in the two ways of testing ("vitamin-free casein," dextrinised rice starch, salt mixture, agar-agar, vitamins B_1 and B_2 as independent preparations or as yeast, and cod-liver oil) whether they began with a shortage of vitamin A as in a vitamin A test or of vitamin B as in a vitamin B test. The former, "vitamin A way," has given us more animals for this work because the tests have necessarily been extended over longer periods (preparatory and test) than in the "vitamin B way," and the rats have apparently exhausted their reserves of the unknown factor. In the "vitamin B way" the rats have a very short preparatory period (10 days) and often a short test period (3–4 weeks), so that the test is often completed before the animals' reserves of the factor are exhausted.

The importance of supplying this factor in the basal diet of all rats used for growth experiments is obvious. It is a simple matter to give "light-white" or a similar sample of casein in the diet of rats preparing for a vitamin B test, for this form of casein is free from either B_1 or B_2. For vitamin A testing, however, a method of removing vitamin A without removing the unknown factor must be adopted. These points will be the subject of a later paper.

Discussion.

Certain food substances have been found to restore growth in rats which had ceased to grow although receiving a diet considered adequate in all factors known to be essential for growth.

It would appear probable that these substances supplied some factor which was missing from the basal diet of the rat, and that those rats which had succeeded in growing to maturity on this diet had reserves of the unknown factor which they had stored from pre-experimental feeding. No satisfactory

extract has yet been obtained from "light-white casein" (B.D.H.), but alcohol and ether extracts of the factor have been prepared from wheat embryo. The fact that it can be so extracted is evidence of its character as a fat-soluble factor.

Another suggestion is that the casein in general use in the vitamin-free diet has, by the process of freeing it from vitamin A, been damaged in some way that has rendered it less digestible or less adequate for growth and that the substances which have restored growth contain "unspoilt" protein. That the active substance can be extracted at all seems to be against this explanation. The fact, too, that many rats grow rapidly on the diet containing the "vitamin-free casein" (Glaxo) shows that it is an adequate protein for some rats at least. Indeed, many rats of both the albino and the black and white strains obviously have been able to digest the protein all their lives. The possibility of racemisation of the protein must be discarded as the activity has been almost completely removed from the protein by hot alcoholic extraction. The fact that the response to the untreated protein is sometimes delayed for many days also seems to be indicative of some actual deficiency rather than of some inability to digest the protein available.

Distinction from vitamin E. At one time it began to appear probable that the factor causing the remarkable resumption of growth would prove to be vitamin E. Its distribution was apparently similar to that of the latter factor; but further work, particularly with wheat-germ oil, makes this at least doubtful. Evans [1928] has demonstrated the growth-promoting power of this factor, and reported that wheat-germ oil in general made a marked difference in growth rate, in the absence of other sources of vitamin E, in a dose of 0·12 g. daily. Three samples which were prepared specially for this laboratory by extraction of wheat germ with light petroleum and concentration *in vacuo* were found to have distinctly less growth-promoting activity than that reported by Evans for vitamin E. One sample prepared by ourselves also showed less activity than was generally experienced by Evans, a dose of 0·16–0·24 g. being necessary to cause a really definite resumption of growth. Moreover, Evans has shown that etiolated pea-shoots are as rich in vitamin E as green ones. Our experience of one species of etiolated shoot (wheat) shows it to be poor in the unknown factor. Further, the liver which we have examined is richer than ox muscle, a relationship which is the reverse of Evans's experience with vitamin E.

The fact that the unknown factor can be destroyed by heat also makes it clear that it is not vitamin E. Moreover, rats suffering from a shortage of it show none of the usual cyclical changes in the reproductive organs as seen by examining the vaginal smears (unpublished work), whereas a shortage of vitamin E has no influence on the oestrous cycle [Evans and Burr, 1927].

There has, moreover, been no indication of a shortage of vitamin E among the animals of the stock colony of this laboratory. (They have had liberal supplies of fresh watercress twice a week, for which we are indebted to

W. Bedford, Esq. of Bere Regis.) Every mating is recorded; each doe is weighed once a week, and oftener during the last week of pregnancy. In the history of some 200 breeding does the occurrence of resorptions has been only about 1 % of the matings which have themselves averaged 4–6 per animal. This factor, then, seems to bear no relation to vitamin E beyond a somewhat similar distribution.

Summary.

Rats often cease to grow even though they are receiving a diet hitherto considered to be satisfactory in calorific value, salt content, and digestibility and also known to contain abundance of all recognised vitamins.

Resumption of growth in these animals was first obtained by the substitution of "light-white casein" (B.D.H.) for the "vitamin-free casein" (Glaxo) generally used in the basal diet.

Other substances which bring about rapid resumption are fresh milk, lettuce, fresh and dried grass, ox muscle, liver and wheat embryo.

Less rapid growth is brought about by watercress and milk which has been simmered for 15 minutes.

Butter and etiolated wheat shoots show very little influence on the growth of these rats.

The power to cause growth resumption is removed from "light-white casein" only with difficulty. Heat can destroy it, at least partially. Cold alcohol, ether, and 2 % acetic acid give inactive extracts and leave unimpaired casein. Boiling alcohol and ether give slightly active extracts and leave definitely impaired casein. The loss may be due to heat.

Boiling alcohol (90 %) and ether give extracts of wheat embryo of definite activity and leave the wheat embryo with only slight activity.

The results here described indicate either the existence of a growth-promoting factor hitherto unrecognised or a biological inadequacy of the protein supplied to the rats in the basal diet. The preparation of active alcohol and ether extracts supports the former view.

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Addendum.

Since this paper was written, we have read that Evans and Burr (*Proc. Soc. Exp. Biol. Med.* 1927, **24**, 740) have demonstrated a deterioration in their 'casein' after long extraction with alcohol and ether.

LXXX. VITAMIN D IN ERGOT OF RYE.

By EDWARD MELLANBY, ELLA SURIE
and DOUGLAS CREESE HARRISON.

From the Pharmacology Department, University of Sheffield.

(*Received May 22nd, 1929.*)

In the course of experiments carried out with another object it was observed that ergot of rye had a powerful action in promoting calcification of the bones when added to diets which, in themselves, resulted in the development of rickets [Mellanby, Surie and Harrison, 1928]. It was therefore decided to investigate this problem in more detail, to study the properties of the calcifying substance in ergot and to consider its relation to or identity with vitamin D.

Experimental methods.

The animals used in this work were puppies, and the technique was that developed by one of us and described elsewhere [E. Mellanby, 1919, 1921]. It consists essentially in giving young animals of the same litter, from 6 to 8 weeks old, a mixed diet which alone produces rickets. Some are given in addition the substances whose effects on bone development are under investigation and the results on the bones are observed in the test and control animals. Since litters of puppies vary to some extent in their reaction to the basal diet, it is important that in examining the results comparison should be made between the animals in each litter and not between animals in different litters. This caution is also necessary because in the experiments to be described there are variations in the basal diets of different litters so that these diets are sometimes more and sometimes less rickets-producing.

Ergot itself can only be administered to dogs in relatively small quantities owing to its unpleasant taste: even when given in such quantities as from 2 to 5 g. daily the animals often refuse to eat their food, so that it is necessary periodically to omit the substance from the diet. In some experiments the powdered ergot was mixed with bread or meat and administered in pill form in the evening, but even so the food was sometimes refused the following day. In the majority of experiments, therefore, the puppies eating ergot did not grow as well as the other members of the family, a fact which must be taken into account in interpreting nutritional experiments. In spite of these difficulties, however, the antirachitic action of ergot is very evident. When alcoholic and ether extracts of ergot replace the ergot itself the difficulties regarding the food intake are avoided; the animals grow at the same rate as the controls and the results are therefore more satisfactory.

The calcifying action of ergot.

The following sample experiment shows that 2 to 5 g. of ergot added daily to a diet which alone produces rickets assist the calcification processes and tend to prevent rickets.

Exp. 1. Two puppies of one litter were given a rickets-producing basal diet: 1302 received the basal diet only; 1305 was given in addition 2 to 5 g. of powdered ergot.

Age at beginning: 7 weeks.

Duration of experiment: 19 weeks.

Basal diet: 100–160 g. bread, 20 g. lean meat, 20 g. separated milk powder, 10 cc. olive oil, 2·5 g. dried yeast, 3 cc. orange juice, 1 g. sodium chloride.

No. of animal	Variations in diet	Initial weight g.	Weight at time of X-ray (19 wks.) g.	Gain wt. in 19 wks. g.	Clinical and radiographic diagnosis of bone condition
1302	Basal diet	2400	6985	4585	Bad rickets (Plate II, fig. 1)
1305	Basal diet + 2 to 5 g. of ground ergot	2510	5820	3310	Normal: no rickets (Plate II, fig. 2)

It is evident that the addition of 2 to 5 g. of ergot daily to the basal diet has prevented rickets.

The solubility of the calcifying agent in alcohol.

Exp. 2. 800 g. of ground ergot were extracted twice with 2400 cc. of absolute alcohol. In the first extraction the mixture was boiled for 1 hour and in the second for ½ hour. The extracts were filtered hot and the alcohol was removed from the combined filtrates under reduced pressure, leaving an oily residue.

Four puppies of the same litter were given a rickets-producing basal diet: 1388 received the basal diet only; 1386 was given in addition the alcoholic extract representing 2 to 4 g. of ergot daily; 1387 was given in addition the residue of ergot after extraction with alcohol, equivalent to 2 to 4 g. of ergot daily; 1389 was given in addition 2 to 4 g. of ergot daily.

Age at beginning: 6 weeks.

Duration of experiment: 11 weeks.

Basal diet: 70–80 g. oatmeal, 20–30 g. separated milk powder, 20 g. lean meat, 10 cc. olive oil, 2·5 g. dried yeast, 3 cc. orange juice, 2–3 g. sodium chloride.

No. of animal	Variations in diet	Initial weight g.	Weight at time of X-ray (11 wks.) g.	Gain wt. in 11 wks. g.	Clinical and radiographic diagnosis of bone condition
1386	Basal diet + alc. ext. of 2 to 4 g. ergot	1725	4720	2995	Rickets (much less than in 1387 and 1388)
1387	Basal diet + ergot residue after alc. extraction	1630	4160	2530	Very bad rickets
1388	Basal diet only	1415	3450	2035	Very bad rickets
1389	Basal diet + 2 to 4 g. ergot	1790	3400	1610	Rickets (much less than in 1387 and 1388)

It is evident that most of the calcifying action of the original ergot is present in the alcoholic extract and that the residue after extraction is devoid of any calcifying effect. The fact that oatmeal forms the cereal in this experiment probably accounts largely for the fact that 1386 and 1389 both have some rickets.

The calcifying effect of the alcohol- and ether-soluble fraction after saponification.

Exp. 3. The oily product obtained from ergot by alcoholic extraction as described in Exp. 2, was well shaken with 500 cc. ether, the insoluble resin being ground up several times with ether to ensure the removal of its ether-soluble contents. The ethereal solution was then filtered and the ether distilled off. This oily residue was dissolved in 100 cc. hot alcohol. 15 g. KOH in 15 cc. water were added and the mixture was boiled under a reflux condenser for 1 hour. The solution was then neutralised with dilute HCl. Portions of this mixture equivalent to 4 g., later to 2 g., of the original ergot were added to the diet of 1407.

Two puppies of the same litter were given a rickets-producing diet: 1406 received the basal diet only; 1407 was given in addition the solution from 2 to 4 g. of ergot after saponification.

Age at beginning: 9 weeks.

Duration of experiment: 10 weeks.

Basal diet: 70 g. oatmeal, 20–30 g. separated milk powder, 20–10 g. lean meat, 10 cc. olive oil, 2·5 g. dried yeast, 3 cc. orange juice, 2–3 g. sodium chloride.

No. of animal	Variations in diet	Initial weight g.	Weight at time of X-ray (10 wks.) g.	Gain wt. in 10 wks. g.	Clinical and radiographic diagnosis of bone condition
1406	Basal diet	2435	4460	2025	Very bad rickets
1407	Basal diet + solution from 4 to 2 g. of ergot after saponification	2145	4320	2175	Almost normal

It is evident that the calcifying factor of ergot is soluble in ordinary ether and is stable to the treatment which saponifies the fats.

Extraction of the unsaponifiable fraction. Having found that the calcifying factor in ergot was ether-soluble and not destroyed by saponification, it remained to see whether it was, like vitamin D, an unsaponifiable substance which could be removed by the ordinary methods for separating this substance from the saponified product.

Our earlier experiments appeared at first to negative this supposition, for the unsaponifiable fraction as then prepared had little or no antirachitic action. The saponification of the oily fraction was carried out as described in Exp. 3. The product was then diluted with its own volume of water and extracted three times with approximately 750 cc. of light petroleum (B.P. 60–80°). The light petroleum was evaporated off and the unsaponifiable residue added to the diet of dogs to test its calcifying properties. Since this is the ordinary method of preparing fat-soluble vitamins from cod-liver oil, it was surprising to find little or no evidence that the calcifying substance had been extracted by the light petroleum.

In subsequent experiments the extraction was made by ordinary ether instead of light petroleum and definite evidence of the presence of the calcifying substance in the unsaponifiable fraction was then obtained, although the activity of the extract did not represent the full activity of the original ergot. Two experiments, one of a preventive and the other of a curative nature, are given as examples to illustrate the calcifying action of the unsaponifiable substance obtained from the fat-soluble fraction of ergot.

The calcifying effect of the unsaponifiable fraction extracted from the saponified fats by ether.

Exp. 4 (*preventive experiment*). Two puppies of the same litter were given a rickets-producing diet: 1497 received the basal diet plus unsaponifiable substance from 4 g. ergot; 1501 received the basal diet only.

Age at beginning: 7 weeks.

Duration of experiment: 12 weeks.

Basal diet: 80–150 g. white flour, 20 g. separated milk powder, 20 g. lean meat, 10 cc. olive oil, 2·5 g. dried yeast, 3 cc. orange juice, 2–3 g. sodium chloride.

No. of animal	Variations in diet	Initial weight g.	Weight at time of X-ray (12 wks.) g.	Gain wt. in 12 wks. g.	Clinical and radiographic diagnosis of bone condition
1497	Basal diet + unsapon. subs. from 4 g. ergot	2120	5100	2980	Very little rickets (Plate II, fig. 3)
1501	Basal diet	2650	5820	3170	Very bad rickets (Plate II, fig. 4)

It is clear that the unsaponifiable fraction prepared in this way has prevented in 1497 the very defective calcification seen in 1501.

Exp. 5 (*curative experiment*). Two puppies of the same litter were given a rickets-producing diet. After 10 weeks' feeding, when both had developed very bad rickets, the unsaponifiable substances representing 6 g. of ergot were added daily to the diet of 1517, that of 1519 being continued without alteration.

Age at beginning: 7 weeks.

Duration of experiment: 17 weeks.

Basal diet: 100 to 150 g. bread, 20 g. separated milk powder, 20 to 10 g. lean meat, 10 cc. peanut oil, 2·5 g. dried yeast, 3 cc. orange juice, 1 g. sodium chloride.

No. of animal	Variations in diet	Initial weight g.	Weight at time of X-ray (10 wks.) g.	Weight at time of X-ray (17 wks.) g.	Clinical and radiographic diagnosis of bone condition	
					After 10 wks.	After 17 wks.
1517	Basal diet for 10 wks., then unsap. residue from 6 g. ergot for further 7 wks.	2185	4700	5520	Very bad rickets similar to 1519	Healing rickets
1519	Basal diet for 17 wks.	2095	4550	4700	Very bad rickets similar to 1517	Very bad rickets

In this case, the unsaponifiable residue has brought about healing. Puppy 1517 was active to some extent at the end of the experiment, 1519 was paralysed.

Having obtained evidence that the calcifying action of ergot was due to some substance with properties similar to those of vitamin D, we have assumed that the active substance is vitamin D since, so far as is known, this is the only entity which controls the processes of calcification in the body. The problem then arose as to how vitamin D was formed in ergot. Since ergosterol was first discovered as a constituent of ergot by Tanret [1889], and since ergosterol is now known to be transformed into vitamin D by ultra-violet radiation [Rosenheim and Webster, 1927; Hess and Windaus, 1927], the first suggestion as to the mode of origin of vitamin D in ergot was that it was

produced at some stage in the growth of the infected rye by the direct action of the ultra-violet radiations of sunlight on ergosterol. This mode of origin did not, however, appear very probable because ergot of rye has a dark bluish-black covering and there was doubt as to whether this covering was permeable to the radiations. If it were permeable, then, since ergot contains abundant unactivated ergosterol, exposure of the ergot grains to sunshine ought to increase its calcifying activity. In order to test this point the following experiment was carried out.

The effect of irradiating ergot with sunlight and mercury-vapour light.

Exp. 6. A sample of whole ergot grains was scattered sparsely over a tin dish and exposed to bright summer sunshine for 12 hours. Another sample was placed on a similar dish and exposed to a mercury-vapour lamp at a distance of about 2 ft. for half an hour. Each sample was then ground and extracted with alcohol. The alcohol was distilled off from each under diminished pressure and the residues were administered to two dogs on an otherwise poor calcifying diet. The third dog received the alcoholic extract of 2 g. untreated ergot.

Three puppies of the same litter were given a rickets-producing diet: 1498 received the basal diet plus alcoholic extract of ergot irradiated by sunlight; 1499 received the basal diet plus alcoholic extract of ergot irradiated by mercury-vapour light; 1496 received the basal diet plus alcoholic extract of untreated ergot.

Age at beginning: 7 weeks.

Duration of experiment: 12 weeks.

Basal diet: 80–150 g. white flour, 20 g. separated milk powder, 20 g. lean meat, 10 cc. olive oil, 2·5 g. dried yeast, 3 cc. orange juice, 2–3 g. sodium chloride.

No. of animal	Variations in diet	Initial weight g.	Weight at time of X-ray (12 wks.) g.	Gain wt. in 12 wks. g.	Clinical and radiographic diagnosis of bone condition
1496	Basal diet + untreated ergot (alc. ext. 2 g.)	2430	5300	2870	Rickets similar to 1498
1498	Basal diet + sunlight ergot (alc. ext. 2 g. ergot)	2400	5500	3100	Rickets similar to 1496
1499	Basal diet + Hg-vapour light ergot (alc. ext. 2 g. ergot)	2150	5500	3350	Slight rickets, definitely less than 1496 and 1498

The alcoholic extract of 2 g. only of non-irradiated ergot was given to 1496 so that some degree of rickets should develop, thus allowing the detection of an increase (if any) in the vitamin D after irradiating ergot by sunlight (1498) and by the mercury-vapour lamp (1499). The results of the experiment show (1) that exposure of intact ergot grains to strong sunlight for 12 hours does not increase their calcifying activity and (2) that exposure to the mercury-vapour lamp for half an hour slightly increases the activity. It is evident that the covering of the ergot grains is relatively impermeable even to very abundant ultra-violet radiations. This result raised some doubt as to whether the presence of vitamin D was due to the direct action of sunlight on the ergosterol present in the ergot.

It appeared desirable, then, to decide whether the vitamin D is primarily a product of the sclerotium or whether the rye embryo in which the fungus

develops is responsible to any extent for the presence of the vitamin. If the fungus and its sclerotium were the responsible agent, there was a possibility that other types of fungi would contain vitamin D. To test this point an experiment was made to see whether the ordinary edible mushroom (*Agaricus campestris*) had any calcifying action. It may be mentioned that Gérard [1892, 1895] found ergosterol not only in ergot of rye but in the whole group of Cryptogams—Basidiomycetes, Myxomycetes, Ascomycetes, Oomycetes and lichens. It seemed possible, therefore, that mushrooms might contain the calcifying vitamin. In the one experiment carried out this did not prove to be the case.

In view of the negative result we then turned our attention to rye germ. The specimens used, so far as we know, were not ergotised. Two samples were tested, one obtained from millers in Belgium, and the other from Germany. Both samples looked uniform in composition and microscopic examination did not reveal any evidence of the presence of mycelial threads. In the following experiment, which shows that rye germ itself has slight calcifying properties, the germ used was of German origin.

The effect of rye germ on calcification.

Exp. 7. Four puppies of the same litter were given a rickets-producing diet, in addition to which 1505 received white flour only; 1506 received a mixture of 80 % white flour and 20 % rye germ; 1510 received 80 % white flour and 20 % rye germ which had been extracted with light petroleum; 1504 received 100 % white flour and the light petroleum extract of rye germ equivalent to that used for 1506.

Age at beginning: 6 weeks.

Duration of experiment: 12 weeks.

Basal diet: 25 g. separated milk powder, 20 g. lean meat, 10 cc. peanut oil, 2·5 g. dried yeast, 3 cc. orange juice, 2 g. sodium chloride.

No. of animal	Variations in diet	Initial weight g.	Weight at time of X-ray (12 wks.) g.	Gain wt. in 12 wks. g.	Clinical and radiographic diagnosis of bone condition
1504	White flour 100 % + light petroleum ext. of rye germ	2625	6220	3595	Slight rickets, less than 1505
1505	White flour 100 %	2650	6250	3600	Rickets
1506	White flour 80 % + rye germ 20 %	2585	6320	3735	Rickets, rather more than 1505
1510	White flour 80 % + rye germ after extraction with light petroleum 20 %	2505	6200	3695	Bad rickets: more than 1506

This experiment shows that the light petroleum extract of rye germ contains some calcifying factor. It will also be noticed that rye germ itself does not bring about the improvement in calcification produced by its extract. The reason for this is seen in animal 1510, for, in this case, when the fraction of the rye germ soluble in light petroleum has been removed, the residue actually makes the condition of the bones worse. In other words, rye germ,

as has been previously shown by E. Mellanby to be the case with wheat germ, contains a rickets-producing factor.

The following facts therefore must be taken into account when considering the origin of the vitamin D in ergot.

(1) Rye germ and fungi in general contain abundant ergosterol.

(2) Rye germ, unaffected by ergot, contains a small amount of activated ergosterol (vitamin D).

(3) In the one form of fungus tested (mushrooms) there was no evidence of vitamin D.

(4) There is no evidence that the ergosterol in ergotised rye germ can be activated to vitamin D on exposure of the whole grain to direct sunlight.

The fact that the experiment with mushrooms gave no evidence of the presence of vitamin D indicates that abundant ergosterol can be present in fungi without any of it being in the form of vitamin D. This is also true of some of the cereals, including oatmeal. On the other hand, although Exp. 7 demonstrates the presence of vitamin D in normal rye germ, the amount present is small as compared with that in the ergot. This suggests that some of the ergosterol in the fungus *Claviceps purpurea* (ergot) is in the active form. How this ergosterol becomes activated to vitamin D is an unsolved problem. Although at the present time the ultra-violet irradiation of ergosterol is the only known mode of origin of vitamin D, it is possible that vitamin D can be made from ergosterol by the growing plant independently of ultra-violet radiations.

Since vitamin D is found so rarely in nature in any quantity, it is interesting that ergot should be one of its sources. So far as is known, fish and egg-yolk are the only substances which are powerfully calcifying in their action, other substances like milk, animal fats and meat containing smaller quantities of vitamin D. Some samples of ergot may be roughly estimated to contain about one-eighth to one-quarter the calcifying activity of cod-liver oil. All samples of ergot so far tested, whether of Russian or Spanish origin, are definitely antirachitic.

Summary.

1. Ergot of rye is a powerful stimulus to calcification of bone. The factor responsible for this action is soluble in alcohol and ether, resists saponification and has the properties, so far as these are known, of vitamin D.

2. Rye germ itself, unaffected by the ergot fungus *Claviceps purpurea*, contains a small quantity of calcifying substance which can be extracted by light petroleum.

3. The irradiation of unground ergot grains by strong sunlight produces no increase and the irradiation by the mercury-vapour lamp only a slight increase in the calcifying activity of ergot, although there is abundant ergosterol present.

Experiment 1

Fig. 1. Fig. 2.

Experiment 4

Fig. 3. Fig. 4.

Fig. 1. *Exp.* 1 (1302). Basal diet only. Severe rickets after 19 weeks of diet.
Fig. 2. *Exp.* 1 (1305). Basal diet + 2 to 5 g. ergot. Bones normal.
Fig. 3. *Exp.* 4 (1497). Basal diet + unsaponifiable substances of the fat-soluble fraction of 4 g. ergot. Very little rickets after 12 weeks of diet.
Fig. 4. *Exp.* 4 (1501). Basal diet. Very bad rickets after 12 weeks of diet.

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LXXXI. HORMONES OF THE ANTERIOR PITUITARY LOBE.

By LESLIE FRANK HEWITT.

From the Hale and Dunn Clinical Laboratories, London Hospital, E. 1.

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It has been recognised for some considerable time that the anterior lobe of the pituitary gland plays an important part in the organism. Disturbances of growth (gigantism, dwarfism, acromegaly) and the sexual system (dystrophia adiposo genitalis) have been ascribed to faulty functioning of the anterior pituitary.

Evans and Long [1923–1924] were first to obtain from the anterior lobe substances which affected growth and the female reproductive cycle. They found that suspensions of anterior pituitary lobes ground up with saline, when injected intraperitoneally, produced increase in the post-pubertal growth of rats and inhibited the female oestral cycle. This inhibition of oestrus has been confirmed by Bellerby [1928], using an opalescent suspension obtained by grinding up anterior pituitary lobes with alkali, neutralising and freeing from cell debris by centrifuging. These suspensions also interfere with the normal birth mechanism [Teel, 1926]. Clear sterile extracts obtained by filtering the suspensions through a bacterial filter produced increase in growth [Putnam, Teel and Benedict, 1928] but were not carefully examined further.

On the other hand, when fresh anterior pituitary lobes are transplanted into females, immature animals are brought to premature sexual maturity and display oestrus within a few days [Smith, 1927; Zondek and Ascheim, 1927]. This same premature maturity is also produced by injections of urine of pregnancy and has been suggested as a test for pregnancy [Zondek and Ascheim, 1928]. These results are confirmed by Evans and Simpson [1928], who claim that the hormone affecting increase in the rate of growth and inhibition of oestrus is not only different from that producing premature sexual maturity but is definitely antagonistic to it. Further, they state that the hormone producing premature sexual maturity is obtained from anterior lobes of pituitary glands by acid extraction, but they give no experimental details.

Briefly then the following effects are ascribed to the anterior pituitary lobe: (1) growth-promoting and oestrus-inhibiting (alkaline extracts of anterior pituitary lobes); (2) premature sexual maturity-producing (occurring in urine of pregnancy; produced by implantation of anterior pituitary lobes and by undescribed acid extracts of the lobes).

This communication details preliminary attempts to repeat and amplify some of the work described above.

Filtered alkaline extracts.

Ox anterior pituitary lobes were worked up a few hours after death of the animal. The author is deeply indebted to Mr Vallet and Messrs Oppenheimer, Son and Co. for the gift of the glands.

On each occasion 50 ox anterior lobes weighing about 75 g. were ground up with sterile sand in a sterile mortar and a little water was added to make a paste, then 100 cc. of 0·1 *N* sodium hydroxide were added and the mixture was well shaken. After standing in the ice-chest for 18 hours the mixture was carefully neutralised to phenol red by addition of 0·1 *N* hydrochloric acid. The suspension was centrifuged for an hour and the supernatant fluid pipetted off and filtered through a sterile 14 cm. Seitz filter; this filtration was slow and continued in an ice-chest at reduced pressure overnight. In this way a clear yellow or pink-brown liquid was obtained and transferred to 1 cc. ampoules. Such extracts retained their activity when kept in an ice-chest for at least 6 weeks.

1 cc. of each extract was injected daily (Saturdays and Sundays being excepted) intraperitoneally into sexually mature, female white rats weighing from 128–146 g. (no toxic symptoms or abscesses were observed even after 6 months' treatment). Each animal was at first maintained on a separate extract, but later when the growth-promoting properties of each extract had been proved in this way all the animals were treated with the same extracts. The animals were weighed daily and the vaginal smears examined daily. Small cotton-wool swabs, wound on small wire loops, were inserted carefully into the vagina and the smear was transferred to a warmed microscope slide and stained with Sebrazi's stain, being covered with a cover slip and examined whilst the stain was still moist. This method was found most satisfactory for rapid cytology.

When injected the rats immediately began to grow more rapidly. On the average the injected rats grew at the rate of over 12 g. per week whilst control animals never grew at a rate exceeding 6 g. per week, the average growth being about 3 g., and the usual rate of adult animals is much less (0·2–2 g. per week). The actual figures obtained are given in Table I and the weekly growth curves in Fig. 1. It will be seen that one injected rat weighed 300 g. whilst the heaviest control animal did not exceed 180 g.

The animals were not injected at the week-ends and after every week-end a drop in weight of 4 to 16 g. was observed. During Christmas week the animals were not injected for 6 days and the weights fell on an average 23 g. (extremes 21–26 g.); weight was regained immediately injections were continued. Now it is known that when the pituitary gland is removed from tadpoles the thyroid gland atrophies and almost completely disappears, and in acromegaly the thyroid gland is frequently increased in size. This suggests a close relationship between the pituitary and thyroid glands, and it is suggested that the injection of anterior pituitary lobe extracts stimulates the

thyroid gland, and that this stimulation to over-activity persists for a time after discontinuance of injections, thus resulting in increased basal metabolism and hence reducing the weight by increased combustion. Despite this marked effect on growth no cessation of oestral cycles was observed. Vaginal smears demonstrated normal recurrence of oestrus in the female rats.

Table I. *Rat weights.*

Rat No.	Extract No.	October 5	12	19	26	November 2	9	16	23	30	December 7	14	21*	28*	Jan. 5 1929
Injected Series:															
1	5	128	142	159	162	174	192	190	195†	215	232	253	266	242	270
2	6	—	146	156	170	184	199	214	235	248†	266	274	290	268	298
3	7	—	—	137	150	158	177	192	200	209†	230	246	258	232	269
4	8	—	—	—	144	168	185	190	202	213	222†	232	246	225	253
Control Series:															
5	—	102	110	115	118	122	127	128	133	133	141	144	142	149	145
6	—	102	112	119	122	130	137	—	—	—	—	—	—	—	—
7	—	127	131	146	153	160	157	156	158	163	162	169	165	175	182
8	—	142	151	153	156	158	161	162	162	165	166	173	176	—	—
9	—	154	157	161	163	163	167	171	175	163	172	174	174	180	180
10	—	152	162	165	163	165	168	173	—	—	—	—	—	—	—
11	—	140	146	152	160	162	173	175	—	—	—	—	—	—	—

* No injections. † Rats transferred to Extract A 14

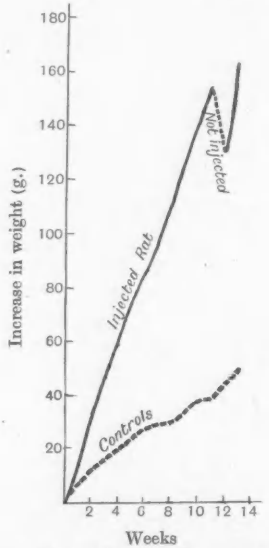

Fig. 1. Mean weekly growth.

In order to facilitate filtration, some of the extracts were rendered definitely acid, filtered and neutralised. This resulted in agglutination of the precipitate and accelerated filtration. The growth-promoting properties of these extracts were, however, variable—one extract promoted good growth

whilst the next did not. The ovaries of animals treated with it were, however, filled with corpora lutea and resembled the so-called "mulberry ovary." These acidified alkaline extracts, therefore, possessed little growth-promoting activity but had a follicle-ripening and corpus luteum-producing effect.

Filtered lime extract.

50 fresh ox anterior lobes were ground up with sand and 300 cc. of saturated lime water were thoroughly mixed with the mash. After standing overnight carbon dioxide was led in and the mixture was shaken until neutral to phenol red. After a further adjustment of the neutrality after standing, the mixture was centrifuged rapidly for 20 minutes and the clear red supernatant fluid was filtered through a sterile Seitz filter. The filtration proceeded rapidly and was complete within a few minutes. This fluid was injected intraperitoneally in 1 cc. daily doses into female rats and was well tolerated. Increased rate of growth was produced with this extract. In one rat the rate of growth for 3 months prior to injection was less than 0·3 g. *per diem*, in the first 23 days after injection the rate had risen to 1·6 g. *per diem*. For a time four other mature rats also received this extract and grew at the increased rate of about 2 g. a day. The oestral cycles were not affected in any animal and proceeded normally. This constitutes a most rapid method of preparing a potent growth-promoting extract without harmful effects to the body-wall (no abscesses produced) but occasionally a potent extract is not obtained.

The solid residue left after extraction, neutralisation and centrifuging was extracted with 100 cc. 0·1 *N* acetic acid. This extract was neutralised and centrifuged yielding an almost colourless clear liquid which filtered rapidly through a small Seitz bacterial filter and yielded an extract which caused increase of growth but no cessation of oestrus. The alkaline extraction, therefore, had not removed all the growth-promoting hormone from the gland paste.

Unfiltered alkaline extracts (*A* 9, *A* 10, *A* 11).

In each case 50 fresh ox anterior lobes were immersed in 40 % alcohol for 10 minutes, rinsed with three changes of 0·9 % saline, ground up with washed sand in a mortar with a little (30 cc.) water and 100 cc. of 0·1 *N* sodium hydroxide; the mixture was allowed to stand in an ice-chest overnight and then neutralised to phenol red with 0·1 *N* hydrochloric acid, all the operations being conducted with precautions for sterility. The neutralised material, which tended always to become more alkaline, was centrifuged rapidly for an hour and the supernatant opalescent pink fluid was mixed with a sterile concentrated aqueous solution containing 1·5 g. of sodium benzoate, and kept in small tubes in an ice-chest. Each day 1 cc. of this suspension was injected intraperitoneally into sexually mature rats. Sterile abscesses tended to form in the abdominal wall, especially when the extracts

remained subcutaneous. The rate of growth was accelerated by these extracts, as shown in Table II. When injections were discontinued an immediate drop in weight after 2 days was observed.

Table II.

		Pre-injection		During injection		Post-injection	
Extract No.	No. of animal	Period days	Mean daily growth g.	Period days	Mean daily growth g.	Period days	Loss in weight g.
9	12	47	0·6	43	1·6	Killed for dissection	
10	13	55	0·7	29	1·7	6	27
11	14	—	—	29	2·1	7	24

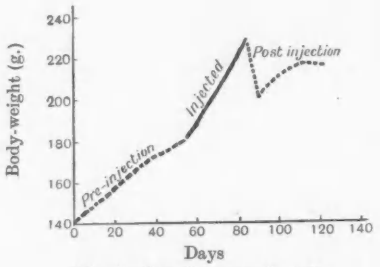

Fig. 2. Growth curve of rat 13.

No oestrus was observed in rat 5 (Extract A 9) during the whole 6 weeks of injections, although the pre-injection period was marked by normal oestral cycles of duration 6, 8, 6, 5, 8, 8, 7 days respectively. With rat 6, oestrus was not affected (Extract A 10), whilst with rat 7 (Extract A 11) the smears were mixed, that is to say, on no occasion were purely cornified epithelial cells alone observed in the daily smear, but the continuous appearance of large quantities of polymorphonuclear leucocytes in the smear, as with rat 5, was absent.

Maturity-producing hormone.

Zondek and Ascheim [1928] have shown that urine from pregnant women contains a hormone which produces premature sexual maturity in mice 3 weeks old. Implantation of fresh anterior pituitary lobes has the same effect [Smith, 1927; Zondek and Ascheim, 1927], but no extract of anterior lobes with these effects has been described in any detail.

Urine of pregnancy. 3 weeks old mice (weighing 6–9 g.) and 3 weeks old rats (weighing 30–40 g.) were injected with 5–6 doses in all, during 3 days, of 0·2–1·0 cc. of catheter specimens of urine (rendered slightly acid) obtained from pregnant women. When the animals were killed on the fifth day evidence was obtained in each case of the production of premature sexual maturity; the uterus was enlarged, the ovaries contained haemorrhagic spots and ripe follicles or corpora lutea and the vaginal smear contained cornified epithelial

cells. In the case of the rats the uteri and ovaries were weighed after dissection, and, whilst in the two control litter-mate sisters the weight of the ovaries and uterus together was in each case 0·06 g., in the rats injected with urine of pregnancy the weights were 0·1–0·15 g. (mean = 0·13 g.).

Anterior pituitary lobe extracts. Attempts were now made to obtain the premature maturity-producing hormone from anterior lobes of pituitary glands. As has been described, the growth-producing hormone is obtained by alkaline or neutral extracts and it was anticipated that acid extracts would contain this maturity hormone.

Acid extract. 50 anterior pituitary lobes were ground up with washed sand and a little water, 100 cc. of 0·1 *N* acetic acid were added and the mixture was allowed to stand in the ice-chest until next morning, when it was centrifuged for 30 minutes. The clear red supernatant fluid was rendered neutral to phenol red by addition of 0·1 *N* sodium hydroxide, and filtered through a sterile Seitz bacterial filter. The filtration was completed within a few minutes. This extract produced considerable increase in rate of growth (*e.g.* an injected adult female rat grew 60 g. in 4 weeks) of mature rats but did not inhibit oestrus. It had no apparent effect on 3 weeks old mice when injected intraperitoneally in 5 doses of 0·1–0·4 cc. in the course of 3 days. The uteri remained thread-like and infantile as distinct from litter-mate sisters receiving injections of urine from pregnant women.

Adsorption. The extraction was conducted as described in the previous section, but after centrifuging the clear red supernatant fluid was shaken thoroughly with pulverised acid-washed kaolin and after standing the suspension was centrifuged and the supernatant fluid decanted. One portion was placed in a tested collodion membrane and dialysed against distilled water for a few hours, and the clear colourless dialysate was filtered through a small sterile Seitz bacterial filter. Another portion was ultrafiltered through fairly thick cellophan, and the colourless ultra-filtrate was filtered through a sterile Seitz bacterial filter.

These two extracts were injected intraperitoneally into females of a litter of 3 weeks old rats, the former in six doses of 1 cc. and the latter in six doses of 0·25 cc. Both these animals showed enlarged uteri and ovaries and cornified epithelial cells in the vaginal smear on the fifth day. Three other females of the litter which received injections of urine from urine of pregnancy also showed the same effects, whilst a control litter-sister remained in the usual infantile condition.

Hydrochloric acid anterior pituitary extract. 50 fresh ox pituitary lobes (80 g.) were ground up with sand in a mortar and made into a paste with 20 cc. of water and 100 cc. of 0·1 *N* hydrochloric acid. The mixture was allowed to stand 18 hours in an ice-chest and then centrifuged rapidly for 30 minutes. The very opalescent supernatant fluid was pipetted off and shaken up with washed kaolin. After standing, the mixture was centrifuged rapidly for 1½ hours. The cloudy supernatant fluid was neutralised with 0·1 *N* sodium

hydroxide (40 cc. were required) and centrifuged for 0·5 hour after standing an hour. The perfectly clear, practically colourless supernatant fluid was decanted and filtered through a Seitz filter in a few minutes. An adult rat injected daily intraperitoneally with 1 cc. of this fluid showed no increase of weight in a fortnight. No appreciable amount of growth-hormone was, therefore, present.

Three female rats, aged 24 days, were taken:

(1) was injected with five doses of 1 cc. of the extract spread over 3 days;

(2) was given five injections of 1 cc. of urine of pregnancy;

(3) was the control.

All were killed on the fifth day with coal-gas. Vaginal smears of (1) and (2) revealed pure cornified epithelial cells indicating oestrus whilst those of (3) did not; (3) possessed a thread-like uterus and undeveloped ovaries whilst (1) and (2) possessed distended large uteri and developed ovaries. The extract therefore possesses, in common with urine of pregnancy, the power of producing premature sexual maturity in young female rats.

Experiments on female rabbits. Extracts of anterior pituitary lobes were made with acetic acid and dilute hydrochloric acid, shaking with kaolin and neutralisation. The sterile filtered extracts were clear and yellow in colour and were injected intraperitoneally in four consecutive daily doses of 2 cc. into healthy female rabbits. The animals were killed on the day after the fourth injection and in every case large clear follicles were found in profusion in the ovaries, many of the follicles being haemorrhagic. The uteri were highly vascularised and pink in colour.

One pregnant rabbit was treated in the same way and the ovaries contained both clear and haemorrhagic Graafian follicles in every stage of development side by side with the corpora lutea of pregnancy.

Discussion.

It has been found possible to obtain extracts of anterior lobes of pituitary glands with the following effects:

(1) growth-promoting (filtered alkaline extracts);

(2) growth-promoting and oestrus-inhibiting (unfiltered alkaline extracts);

(3) premature maturity- and ripe follicle-producing (acid extracts treated with kaolin, etc.).

It would seem therefore that three effects may be produced by extracts of anterior lobes. Evidence from histological, surgical, pathological and biochemical fields presents a strong case for the existence of hormones in the anterior pituitary lobe controlling growth and the female reproductive cycle. Two inferences may be drawn: theoretically, one is faced with the presumption that the anterior pituitary lobe may control the ovarian cycle, and practically, one may hope for the establishment of replacement therapy in disturbances of growth and the female reproductive system.

Discontinuance of injections of growth-promoting extracts is followed by an immediate and rapid fall in weight which reaches its lowest level (a fall of over 20 g. in rats) in about one week. When injections are recommenced weight is rapidly regained. It appears possible that the extract had two effects: (*a*) to stimulate growth, and (*b*) to stimulate the thyroid gland. That the thyroid gland is affected by the anterior pituitary lobes is shown by the fact that ablation of the anterior pituitary lobe in tadpoles is followed by atrophy of the thyroids [Smith, 1922]. Anterior pituitary deficiency results in depression of metabolism, temperature, etc. [Reye, 1928]. Anterior pituitary hypertrophy is frequently associated with increase in the activity of the thyroid.

Possibly growth stimulation ceases directly injections are discontinued, but thyroid stimulation, which is over-weighted by growth stimulation during the injections, continues for a time, increases the metabolic rate and hence causes the loss of weight observed. The possibility arises therefore that anterior pituitary medication might improve the condition of intractable cases of hypothyroidism.

SUMMARY.

1. The preparation is described of extracts of anterior pituitary lobes which (i) promote growth, (ii) inhibit oestrus, (iii) produce precocious sexual maturity and cause ripening of Graafian follicles.

2. When rats, which have been treated with growth-promoting extracts, are no longer injected a rapid fall in weight is observed. A tentative explanation lies in stimulation of the thyroid gland.

This work was carried out under the auspices of the Gibbons Research Fund, and the author is indebted to Dr R. A. Gibbons, Mr Eardley Holland and Dr P. N. Panton for their helpful interest.

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LXXXII. THE RELATION BETWEEN CYSTINE YIELD AND TOTAL SULPHUR IN VARIOUS ANIMAL HAIRS.

By CLAUDE RIMINGTON.

From the Biochemical Department, British Research Association for the Woollen and Worsted Industries, "Torridon," Headingley, Leeds.

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In a recent communication [Rimington, 1929] it was shown that by employing the Sullivan [1926] method for the determination of cystine practically the entire sulphur of wool could be accounted for as cystine. In all, six different varieties of wool and two of Turkey Mohair were examined in this way with identical results.

In the present paper the same method has been applied to the examination of some animal hairs, many of which find important applications in the textile industries.

The results are similar to those obtained when working upon the wools; there is however one notable exception. Specimens of camel hair yield not more than 94 % of their total sulphur as cystine; there remains 6 % unaccounted for, which may represent a sulphur-containing compound other than cystine.

Experimental.

The experimental details of the present investigation differed in no way from those already described [Rimington, 1929]. All samples of hair were carefully degreased in warm benzene and subsequently washed, first in 0·1 % saponin solution and then in distilled water. Moisture and total sulphur were determined by weighing out representative samples of the air-dried materials—two samples for moisture (regain) determinations and two or three for sulphur determination. The loss in weight of the former was recorded when a current of dry air was drawn over the sample contained in a specially designed bottle and maintained at a temperature of 105° until constant in weight.

Total sulphur was determined by a modification of the Benedict-Denis method. It is hoped to publish the details of this modification in the immediate future.

Table I shows the results obtained during the course of a typical experiment, the material in this instance being white alpaca tops, whilst in Table II the results of the whole investigation are summarised.

Table I. *White alpaca tops. Hydrolysis of* 6 *g. by* 20 % *HCl.*

Time from commencement of hydrolysis hours	Cystine in hydrolysate: By Folin and Looney's method g.	Cystine in hydrolysate: By Sullivan's method g.
½	0·9200	0·1000
1	0·8800	0·2070
2	0·8800	0·4163
4	0·8850	0·6450
5	0·8850	0·6975
6	0·8950	0·7480
7	0·8850	0·7863
9	0·8900	0·7969
Total sulphur (determined upon a 5 cc. aliquot part) expressed as cystine		0·7937

$$\text{Therefore } \frac{\text{Total S (hydrolysate)} - \text{cystine (Sullivan)} \times 100}{\text{Total S}} = -0{\cdot}40.$$

The mean moisture content of the alpaca on drying to constant weight was 10·675 % of the wet weight.

Total sulphur determinations:

(i) (Benedict-Denis) 0·2631 g. moist alpaca gave 0·0659 g. $BaSO_4$.
S = 3·44 % of wet weight.

(ii) (Benedict-Denis) 0·2430 g. moist alpaca gave 0·0621 g. $BaSO_4$.
S = 3·41 % of wet weight.

(iii) (Carius) 0·4018 g. moist alpaca gave 0·1026 g. $BaSO_4$.
S = 3·50 %.

Mean S = 3·86 % of dry weight.

Table II.

Material	Total S % dry weight	Cystine in hydrolysate: By Sullivan's method (max. value) g.	Cystine in hydrolysate: By Folin and Looney's method g.	Total S of hydrolysate (as cystine) g.	Ratio*
Dog's hair (white Samoyed)	5·07	1·0400	1·0080	1·0265	−1·31
Rabbit fur (white Angora)	4·59	0·7423	0·8117	0·7399	−0·32
White alpaca tops	3·86	0·7969	0·8860	0·7937	−0·40
Camel hair tops	3·25	0·6065	0·7975	0·6395	+5·16
Camel hair tops	3·25	0·5510	0·6300	0·5908	+6·73
Crossbred 40's wool	3·03	0·7430	0·6690	0·7420	−0·13
Tunisian goats' hair (black)	3·54	0·6792	0·6667	0·6751	−0·61

* $\dfrac{\text{Total S (hydrolysate)} - \text{cystine (Sullivan)} \times 100}{\text{Total S}}$.

Discussion.

These results show clearly that animal hairs, which contain the highest percentage of sulphur found in any of the natural proteins, resemble the wools already examined [Rimington, 1929] in that all this sulphur can be accounted for as cystine except in the one case mentioned below.

The determinations of total sulphur show once more the variability in sulphur content of the hairs of different species. In general the results in this direction accord well with the findings of Barritt and King [1926].

Among the samples examined, camel hair has proved to be exceptional. Reference to Table II will show that in this particular case the extent to which the cystine accounted for fell short of that supposedly present, were the total sulphur of the hair expressed as cystine, was 5·16 % and 6·73 % of the calculated cystine value in two typical experiments. This difference is outside the range of experimental error.

The cause of this anomaly is at present under investigation. The specimens of camel hair used contained a very large proportion of coarse, outer-coat fibres. In the experiments described no attempt was made to remove these.

Summary.

Several varieties of animal hairs have been examined, and, in all cases but one the entire sulphur of the purified hair has been accounted for as cystine.

In the case of camel hair, the cystine value falls short of that required, were all the sulphur present expressed as cystine, by an amount considerably greater than the experimental error.

The author wishes to thank Mr A. T. King for his continued interest and help in the subject of this research.

Note concerning the publication of Marston [1928].

While the first paper of this series [Rimington, 1929] was in the press a paper appeared in which Marston [1928] claimed to have established the fact that the entire sulphur of wool could be accounted for as cystine. In view of the fact, however, that the method of cystine estimation employed by Marston—namely, the colorimetric procedure of Folin and Looney [1922]—was rejected by the present writer on account of its non-specificity, which to his mind rendered it unsuitable when used alone to demonstrate such a point as that now under discussion, it was felt that a few words upon the subject would not be out of place.

Hunter and Eagles [1927] object to the method as originally described by Folin and Looney on account of the development of a blue colour in cystine-free solutions caused by the slow liberation of sulphurous acid. But much more serious is the fact that the Folin and Looney reagent appears to be sensitive to some substance or substances containing sulphur, but not identical with either cystine or cysteine.

Thus du Vigneaud, Jensen and Wintersteiner [1928], in a study of crystallised insulin, found that the hydrolysed product gave a value for cystine by the Folin-Looney reagent equal to the total sulphur but approximately twice as great as that obtained when using the Sullivan colorimetric method.

Sullivan's [1926] method is, so far as is known, absolutely specific for cystine and cysteine and it is this method which has been employed by the present writer in his investigations upon the cystine content of wools and hair. Cystine peptides, such as glutathione, give no colour with the Sullivan reagent whilst they do so with the Folin-Looney reagent.

In view of the work of du Vigneaud, Jensen and Wintersteiner, and since Marston's conclusions are based solely upon observations made with Folin and Looney's reagent, his conclusions must be regarded with some reserve.

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LXXXIII. THE RELATIONSHIP BETWEEN HAEMOLYTIC COMPLEMENT OF GUINEA-PIG SERUM AND LIPASE.

By JOHN GORDON and ARTHUR WORMALL.

From the Department of Pathology and Bacteriology and the Department of Physiology, School of Medicine, University of Leeds.

(*Received June 28th, 1929.*)

The system in serum which is responsible for the haemolysis of sensitised red cells appears to have many of the properties of an enzymic system, and it has been suggested by several authors that this haemolysis is due to the action of an enzyme on integral constituents of the red cell, leading to a rupture of the membrane [cf. Landsteiner, 1929]. Complement and enzymes have many properties in common, but certain facts have been adduced to disprove the view that complement is enzymic in nature. Complement, unlike the enzymes, appears to take part in specific haemolysis according to the law of definite proportions [Liebermann, 1906] and is used up as the reaction proceeds; these facts can probably be explained, however, by a destruction of complement by secondary processes [cf. Liefmann and Cohn, 1911] and, indeed, a similar inactivation occurs in many enzymic reactions.

The investigations described here were concerned with (*a*) the general relationship between the complement power and the esterase activity of guinea-pig serum and heated guinea-pig serum (56° for 30 mins.); (*b*) the lipase and esterase activity of guinea-pig serum which has been inactivated for haemolytic power by treatment with zymin, which inactivates the third component, or with ammonia which inactivates the fourth component; (*c*) the influence of the amboceptor on the lipase and esterase activities of guinea-pig serum; and (*d*) the esterase activity of a special protein preparation from guinea-pig serum, a preparation which has no complete complement power but retains part of the complement system.

If the action of haemolytic complement can be regarded, according to the lipolytic theory, as the action of a serum-lipase on the fatty constituents of the envelope of the sensitised red cells, then it might be expected that the haemolytic activity would run parallel with the power to hydrolyse fats. The lipase of guinea-pig serum has, however, little or no action on such fats as olive oil, and therefore the lipase (or esterase) activity has to be determined by the use, as substrate, of some ester such as ethyl butyrate. Our experiments, in which the sera of four or five guinea-pigs were compared at the same time, have shown that the two activities, haemolytic power and esterase

power, are not always parallel, although a general parallelism has been observed. In addition, it has been found that guinea-pig serum heated at 56° for 30 mins. has no demonstrable haemolytic action on sensitised red cells, but has a significant esterase action.

Complement can be "split" by dilute acids into two separately inactive fractions, a globulin and an albumin fraction, each of which contains a heat-labile component (possibly of protein nature) and a relatively heat-stable component. It is possible, however, to destroy either of these two relatively heat-stable factors with no significant loss of the other components of the system, and, if the lipase and complement systems are identical, these methods of inactivation—the removal of the third component of complement by treatment of the serum with yeast (or zymin) or the inactivation of the fourth component by ammonia—should produce a diminution or destruction of the lipolytic power. It has been found, however, that zymin-inactivated serum and ammonia-inactivated serum, which have no haemolytic complement action, have lipase and esterase activities equal to those of the original serum. The third and fourth components of haemolytic complement, therefore, are not essential for serum-lipase action.

In view of the suggestions made by other authors that the immune body functions either as a specially active lipase or as a co-enzyme for the serum-lipase, we have carried out investigations to determine the lipase and esterase activities of guinea-pig serum before and after the addition of sufficient immune body to render the serum haemolytic for unsensitised red cells. These experiments have shown that the immune serum used has, in the concentrations necessary for promoting haemolysis, practically no lipase or esterase action, nor does it activate the lipase or esterase of normal guinea-pig serum.

Other investigations which we have been carrying out have been concerned with the rôle played by the proteins of serum in complement action, and it was thought of interest to compare the lipolytic activities of the protein preparations used with those of the original sera. Similar preparations from pig's pancreas, obtained by the method of Hardy and Gardner [1910] and Hartley [1925] with the modification introduced by Hewitt [1927] have proved very useful in lipase and esterase investigations [Dawson and Platt, 1928] (Phillis and Platt, unpublished investigations), and from the results of the last-named authors we expected to find a marked esterase power with our protein preparations from guinea-pig serum. This proved to be the case, and these preparations possess a high percentage of the lipolytic power of the original guinea-pig serum. The preparations have, however, no haemolytic action on sensitised red cells, and thus support is furnished for the view that the action of complement is not due to the action of a simple lipase or esterase system.

The experiments described here indicate, therefore, that there is no simple relationship between haemolytic complement and serum-lipase or -esterase, but there is still the possibility to be considered that this enzyme is one of

the components of the complement system. The experiments with ammonia-treated serum and zymin-treated serum show, however, that no relationship exists between the serum-esterase or -lipase and the relatively heat-stable components of complement, whilst the retention of a significant part of the esterase power, when the serum is heated at 56° for 30 mins., appears to indicate that the lipolytic function cannot be attributed to the heat-labile components since the latter have no appreciable haemolytic activity after this heating.

Experimental.

Guinea-pig serum was used as the source of complement and for the haemolytic tests the serum was diluted accurately with 0·9 % NaCl or, in certain experiments, as described later, with a phosphate-NaCl mixture. To varying amounts of the diluted serum, 1 cc. of a 4 % suspension of sensitised red-cells (ox) and sufficient 0·9 % NaCl to make the total volume 2 cc. were added, and the mixtures were incubated at 37°. The degree of haemolysis after 0·25, 0·5 and 1 hour was recorded as follows:

+ + + +	Complete haemolysis
+ + +, + + and +	Intermediate grades of haemolysis
−	No haemolysis

The esterase activity of a serum was determined by allowing the serum to act on ethyl butyrate in the presence of a phosphate buffer of p_H 7·6. The mixtures were placed in small stoppered bottles and were shaken for 2–4 hours at 37°. The contents of the bottles were then titrated with 0·1 *N* sodium hydroxide to a standard colour with phenolphthalein or thymol blue. Control experiments with serum plus buffer (no ester) were made in every case and toluene was added to each bottle to inhibit changes due to bacterial action. The figures given in the tables below represent the mean of two duplicate determinations.

The "true" lipase activity of guinea-pig serum, or the power to hydrolyse olive oil, is very slight, and the amount of hydrolysis noted is very little greater than the experimental error of the method, but it was thought desirable to make determinations of this nature, especially in those experiments (*e.g.* those with the immune serum) where an increase in this power would be of some significance. The technique was similar to that for the esterase determinations. A neutralised emulsion of olive oil was used as substrate and the mixtures were titrated at the end of the experiment with 0·05 *N* alcoholic KOH after the addition of 5 to 6 volumes of an alcohol-ether mixture (4 volumes of absolute alcohol and 1 volume of ether), thymol blue or thymolphthalein being used as indicator. The amount of hydrolysis was very small even after incubation for 3 days. It was also thought desirable to determine whether any relationship exists between the lecithin-splitting power of the serum and complement. It has been shown, however [Schumoff-Simanowski and Sieber, 1906], that the sera of various animals have no appreciable hydrolytic action on lecithin, and we are indebted to Dr E. R. Dawson for confirming this with the guinea-pig sera which we have used for our tests.

(*a*) *Comparison of the complement and esterase activities of normal serum and those of heated serum.*

Serum was collected from several guinea-pigs and the complement and esterase powers of each serum were determined. A result which is typical of many experiments is recorded in Table I.

Esterase activity.

(*a*) 10 cc. phosphate buffer p_H 7·6 (or NaCl-phosphate solution) plus 1 cc. serum plus 0·5 cc. ethyl butyrate plus 1 cc. toluene.

(*b*) Control—as in (*a*) but no ethyl butyrate.

The NaCl-phosphate solution, which was used in certain experiments in the esterase determinations and used also for diluting the complement for the haemolytic test, consisted of a mixture of equal volumes of 1·35 % NaCl and a phosphate buffer of p_H 7·6 [see Cole, 1926, p. 29]. This mixture was used in many experiments since it rendered possible a comparative measurement of lipolysis and haemolysis with a constant salt and phosphate concentration. The results were always identical with those in which the simple phosphate buffer was used for the esterase (or lipase) determinations and 0·9 % NaCl for the haemolytic tests.

Table I. *Complement activity and esterase activity of different guinea-pig sera.*

	Complement activity: Haemolysis of sensitised cells after 1 hr. by				
Serum	0·10–0·03 cc. of serum	0·025 cc. of serum	0·018 cc. of serum	0·012 cc. of serum	Esterase activity (0·1 *N* NaOH required to neut. the acid produced by hydrolysis in 4 hrs.)
1	+ + + +	+ + + +	+ + + +	+	3·45 cc.
2	+ + + +	+ + +	+	–	4·28 cc.
3	+ + + +	+ + + +	+ + + +	+	4·96 cc.
4	+ + + +	+ +	+	–	4·28 cc.

From the results given in Table I and from those of a large number of experiments in which the conditions of the determinations have been varied, the conclusion is reached that the two functions, the haemolytic complement activity and the esterase activity of guinea-pig serum, do not show that relationship which would be expected if the haemolytic action of complement were due to the action of the serum-esterase on the fats of the red cell.

Since haemolytic complement is readily inactivated when guinea-pig serum is heated at 56° for 0·5 hour, it was thought of interest to determine the influence of this treatment on the lipase and esterase of the serum. Jobling and Bull [1913] were unable to obtain a lipase-free complement by heating the serum and found that the lipase was somewhat more resistant to heat than complement.

The results of typical experiments are given in Table II, and they show that, whereas the heating process destroys all the measurable complement activity of the serum, a significant part of the esterase activity is retained;

in addition, no detectable diminution of the slight "true" lipase activity of the serum occurs as a result of the heating process.

Esterase activity.

(*a*) 2·0 cc. serum plus 10 cc. phosphate buffer p_H 7·6 (or NaCl-phosphate solution) plus 0·5 cc. ethyl butyrate plus 0·5 cc. toluene.

(*b*) Control (no ethyl butyrate).

Lipase activity.

(*a*) 4·0 cc. serum plus 4 cc. phosphate buffer p_H 7·6 (or NaCl-phosphate solution) plus 1 cc. neut. emulsified olive oil plus 1 cc. toluene.

(*b*) Control—no olive oil.

(*c*) Control—olive oil plus phosphate buffer plus toluene.

Control (*c*) showed that a very slight hydrolysis of the oil occurred even in the absence of serum, and a correction for this hydrolysis has been made in all the experiments with olive oil.

Table II.

Serum	Esterase activity (cc. 0·1 *N* NaOH)			Lipase activity (cc. 0·05 *N* alc. KOH)			Complement activity (amounts of serum as in Table I)
Normal	3·56	3·50	5·26	0·25	0·14	0·18	Good (as in Table I)
Heated (56° for 30 mins.)	0·85	0·70	1·42	0·27	0·16	0·14	None
Incubation period (in hrs.) for lipase determinations	2½	2	4	44	70	72	—

(*b*) *The esterase and lipase activities of NH_3-inactivated serum and zymin-inactivated serum.*

The "third component" of serum, a relatively heat-stable component, is inactivated when the serum is treated with yeast [Coca, 1914; von Dungern, 1900; Ehrlich and Sachs, 1902] or zymin [Whitehead, Gordon and Wormall, 1925]. Another component is destroyed or inactivated by small amounts of ammonia [Gordon, Whitehead and Wormall, 1926] and zymin-inactivated serum and NH_3-inactivated serum are each devoid of complement activity. Experiments have been made to determine the effect on the esterase and lipase of the serum of destruction of these two components, and it has been found (cf. Table III) that the three sera concerned—untreated guinea-pig

Table III. *Lipase and esterase activities.*

Cf. details for Table II. The period of incubation for these determinations was 2–4 hrs. for the esterase and 48–72 hrs. for the lipase determinations.

Serum	Esterase activity: cc. of serum used	cc. 0·1 *N* NaOH			Lipase activity: cc. of serum used	cc. 0·05 *N* alc. KOH			Complement power (amounts as in Table I)
Normal	0·4	2·40	—	3·2	4·0	0·20	0·20	0·18	Good
	2·0	3·40	4·10	5·0					(as in Table I)
Zymin-inactivated	0·4	2·40	—	3·1	4·0	0·24	—	0·15	None
	2·0	3·40	3·85	4·9					
NH_3-inactivated	0·4	2·40	—	3·0	4·0	0·12	0·15	0·20	None
	2·0	3·70	3·90	5·1					

serum, zymin-treated serum and NH_3-treated serum—have approximately the same esterase power. The three sera have also the same "true" lipase activity, although this activity, as mentioned above, is extremely weak compared with the esterase activity. These results indicate, therefore, that the two relatively heat-stable components are essential for haemolytic complement action but not for the action of the esterase or lipase of guinea-pig serum.

(*c*) *The influence of the immune serum on the esterase and lipase activities of guinea-pig serum.*

Sufficient immune serum (anti-ox-erythrocyte serum) was added to guinea-pig serum to render the mixture haemolytic for unsensitised ox red-cells and the lipase and esterase powers of this mixture were compared with those of the guinea-pig serum. The results of these experiments (Table IV) indicate that the immune serum (previously heated at 56° for 30 mins. to destroy the complement present) has no appreciable lipolytic activity in the amounts used, nor has it any activating action on the esterase or lipase of guinea-pig serum.

Esterase activity.

Series (*a*). A. 0·4 cc. guinea-pig serum plus 10 cc. NaCl-phosphate buffer p_H 7·6 plus 0·5 cc. ethyl butyrate.

B. 0·4 cc. guinea-pig serum plus 10 cc. NaCl-phosphate buffer p_H 7·6 plus 0·5 cc. ethyl butyrate plus 0·025 cc. inactivated immune serum.

C. 0·4 cc. 0·9 % NaCl plus 10 cc. NaCl-phosphate buffer p_H 7·6 plus 0·5 cc. ethyl butyrate plus 0·025 cc. inactivated immune serum.

Series (*b*). The same as series (*a*) with 5 times the amount of guinea-pig and immune sera. Controls, with no ester, were made for all these determinations, and 0·5 cc. of toluene was added to each solution. The mixtures were shaken at 37° for 2–4 hours and were then titrated with 0·1 *N* NaOH, phenolphthalein being used as indicator.

Lipase activity.

Amounts of serum and buffer as for Table II—incubation period, 72 hours.

Table IV.

Serum		Esterase activity (cc. 0·1 *N* NaOH required)			Lipase activity (cc. 0·05 *N* alc. KOH)		Haemolysis of unsensitised ox-cells
Normal guinea-pig serum (A)	(*a*)	3·22	2·36	2·60	0·10	0·10	None
	(*b*)	5·26	3·56	4·42			
Guinea-pig serum + immune serum (B)	(*a*)	3·36	2·38	2·70	0·13	0·09	Good
	(*b*)	5·00	3·75	4·40			
Immune serum (C)	(*a*)	0·06	0·06	0·05	0·0	0·03	None
	(*b*)	0·14	0·08	0·05			

(*d*) *Esterase activity of a protein preparation from guinea-pig serum.*

The proteins of guinea-pig serum were precipitated with a mixture of alcohol and ether at low temperatures by the method of Hardy and Gardner [1910] as used by Hartley [1925] and stable, finely divided powders obtained. Similar preparations obtained by treating serum with alcohol at low temperatures were found by Dean [1913] to have no end-piece of complement and our preparations, in a similar manner, showed no complete complement power. These preparations retained, however, a large part of the esterase activity of the original serum and thus possession of esterase activity is not sufficient to endow the preparation with full complement power.

Experimental details.

A. 5 cc. of guinea-pig serum plus 36 cc. of cold alcohol-ether mixture [cf. Hartley, 1925]. The protein preparation was extracted continuously in a Soxhlet apparatus for 20 hours and then dried *in vacuo*. The powder was dissolved in 4·5 cc. of distilled water and made up to a volume of 5·0 cc. with sufficient NaCl to give 1 % NaCl in the final solution.

B. The same guinea-pig serum plus toluene, kept at room temperature.

C. The same guinea-pig serum, kept in the ice-box.

D. Fresh guinea-pig serum.

The esterase determinations were made as before, with phosphate buffer (p_H 7·6) or, in some experiments, the NaCl-phosphate buffer mixture. Series (*a*) with 0·4 cc. serum and series (*b*) with 1·0 cc. serum.

Table V. *The esterase activities of a protein preparation and of preserved and fresh sera.*

Serum	Esterase activity (cc. of 0·1 *N* NaOH)		Complement activity Haemolysis of 1 cc. of sensitised cells after 1 hr. by		
			0·1 cc. of serum	0·05 cc. of serum	0·02 cc. of serum
A. Protein preparation	1·66	1·54	–	–	–
	2·34	2·00			
B. Preserved serum	2·82	1·86	+ + + +	+ + + +	+ +
	3·54	2·44			
C. Preserved serum	2·76	1·86	+ + + +	+ + + +	+ + +
	3·70	2·50			
D. Fresh serum	2·96	—	+ + + +	+ + + +	+ + + +
	4·1	—			

Summary.

1. The complement activities of various guinea-pig sera tend to run parallel with the esterase activities of these sera, but discrepancies occur which do not support the view that the action of complement is a simple esterase function.

2. Destruction or inactivation of either of the two relatively heat-stable components of complement does not appreciably diminish the esterase activity, and, as far as it is significant, the lipase activity of guinea-pig serum.

3. Guinea-pig serum when heated at 56° for 30 mins. loses all detectable complement action but still retains a distinct esterase power.

4. Protein preparations, obtained by the treatment of guinea-pig serum with alcohol and ether at low temperatures, possess no complete complement power but retain a large part of the original esterase of the serum.

5. The immune body essential for complement action has no influence on the esterase (or lipase) activity of guinea-pig serum when used in amounts sufficient to promote haemolysis of ox red-cells.

6. No evidence has been obtained that the esterase (or lipase) of guinea-pig serum takes part in specific haemolysis. The action of haemolytic complement cannot be ascribed to a hydrolysis of the fatty substances of the red-cell envelope by the powerful esterase or the very weak lipase of guinea-pig serum.

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LXXXIV. STUDIES ON NERVE METABOLISM.

IV. CARBOHYDRATE METABOLISM OF RESTING MAMMALIAN NERVE.

By ERIC GORDON HOLMES and RALPH WALDO GERARD.

From the Pharmacological Laboratory, Cambridge, and the Department of Physiology, University of Chicago.

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In previous papers of this series [Gerard, 1927; Gerard and Meyerhof, 1927], the respiration and lactic acid production of isolated frog nerves, at rest and active, have been considered. An attempt to follow by direct analysis the corresponding changes in carbohydrates was not successful because the methods used were inadequate for the treatment of the small amounts of tissue available. In the present experiments we have had recourse to the much larger nerves obtainable from rabbits. The results are directly comparable to similar studies for mammalian brain at 37° but cannot be accurately compared with those available for amphibian nerve at 15°. On the other hand determinations of oxygen consumption and lactic acid formation for both have helped to bridge the gap.

The present studies are limited to determinations of lactic acid, glycogen and "free carbohydrate" as affected by rest in oxygen and nitrogen.

Methods.

Lactic acid was estimated by two methods; in some experiments the method of Meyerhof [1920] was used, in which the alcoholic extracts of the tissue are deproteinised, and extracted with amyl alcohol; in others, the alcoholic extracts were treated with Schenk's reagent and copper sulphate and calcium hydroxide.

The results obtained by these two methods agree very well. In both cases the final estimation was performed by distillation (without aeration) from the manganous sulphate-sulphuric acid reagent of Friedemann, Cotonio, and Shaffer [1927]. 0·01 *N* permanganate was used, and the "bound" bisulphite liberated with sodium bicarbonate, and titrated with 0·005 *N* iodine. All such estimations were performed in duplicate.

The estimation of "free carbohydrate," *i.e.* carbohydrates other than glycogen, presented considerable difficulty; the amount of tissue was small and the concentration of carbohydrate low, consequently the error due

to non-carbohydrate reducing substances was so large as to invalidate completely any experiments in which it was ignored.

The use of a technique based on the method of Bissinger [1926] offered a way out of the difficulty. The tissue was fixed in iced alcohol, chopped, and ground with sand, and water added to bring the alcohol concentration to 60 %. Next day the alcohol was filtered off through a Jena glass filter (mesh 5–7) and extracted three times with 60 % alcohol. The combined extracts were slowly evaporated on a water-bath; the subsequent procedure depended upon the method chosen for lactic acid estimation. If the amyl alcohol method was contemplated, the extracts were made up to volume (20 cc.) and halved; both halves were evaporated, one being used for lactic acid estimation, the other for carbohydrate: if lactic acid was to be estimated by the Schenk method, no halving of the extract was necessary. The residue was treated with 4 cc. of Schenk's reagent, filtered through asbestos, and the beaker and precipitate were washed with three 1 cc. portions of Schenk's reagent. Hg was removed by H_2S, and H_2S by aeration, washed filter-papers being employed for filtration. (Unwashed filter-paper yields enough reducing substance to introduce an error.)

The carbohydrate was then extracted from the solution as follows.

To the solution (volume about 10 cc.) in a centrifuge tube, was added 1/6 volume of 10 % $CuSO_4$. A few drops of strong sodium hydroxide were sufficient to neutralise the HCl present (shown by commencing precipitation of $Cu(OH)_2$. The tube was placed in ice, and 1/3 volume of 10 % suspension of $Ca(OH)_2$ was added. The tube was left in ice, with occasional shaking, for 30 mins. It was then centrifuged. The supernatant fluid was used, if required, for lactic acid estimation; in that case, both its volume and that of the total contents of the tube were accurately measured. The precipitate was treated with 10 cc. *N* H_2SO_4, then with H_2S, the CuS and $CaSO_4$ were centrifuged, and washed three times with water. After aeration of the fluid and washings, solid Na_2CO_3 was added, until the remaining calcium had been precipitated. The fluid was quickly filtered through a washed filter-paper into a 50 cc. volumetric flask, containing 1 cc. conc. HCl. The precipitate was washed with water, and the contents of the flask made up to volume. 15 cc. portions of the fluid were pipetted out, neutralised, and the reducing substance was estimated by the method of Hagedorn and Jensen [1923].

It is not claimed that this procedure is completely satisfactory. When tested on glucose solutions losses sometimes occur, for which no adequate explanation has been found, and which may amount to 10 % of the amount of glucose added (Table I). No more perfect procedure has, however, been elaborated, and the copper-lime precipitation does afford some assurance of the separation of sugars from the other non-carbohydrate reducing substances in tissue extracts.

Glucose can be recovered by this technique from admixture with creatine and creatinine. Embden and Zimmermann [1924] have shown that hexose-

Table I. *Recovery of glucose (from aqueous solution).*

Glucose added mg.	Glucose recovered mg.	Difference mg.	Error %
1·111	1·048	−0·063	− 5·7
0·666	0·694	+0·017	+ 2·6
0·444	0·420	−0·02	− 4·5
0·192	0·204	+0·012	+ 6·2
0·383	0·350	−0·033	− 8·1
0·575	0·518	−0·057	− 9·9
0·767	0·689	−0·078	−10·2
0·353	0·436	+0·083	+23·0
0·865	0·879	+0·014	+ 1·6
0·240	0·249	+0·009	+ 0·4
0·481	0·428	−0·053	−11·0
0·721	0·719	−0·002	− 0·3

Numerical average error 7 % Algebraic average error −2·3 %

phosphate is carried down on the precipitate, and would therefore be included in our carbohydrate fraction. Apparently creatinine-phosphoric acid is precipitated, even though the free base is not. If creatine-phosphoric acid is present in nerve, it is improbable that it would survive as such until the copper-lime precipitation is carried out, since the "free sugar" fraction always stands for several hours, often overnight, in the acid Schenk reagent. In addition, no creatinine can be detected by the Jaffé or Weyl tests in the final fraction which is used for sugar estimations. (These tests would not show the presence of creatine, but the reducing power of that substance is low.) Finally, the curve obtained by plotting reducing value observed against quantity of fluid taken is linear; in the case of interference by creatinine, the curve falls away as the amount taken for estimation is increased [Holmes and Holmes, 1926]. We therefore feel confident that the values we observed are not distorted by contamination with creatinine.

Glycogen was estimated at first by the modified Pflüger method previously described by one of us [1926]. This was found to be unsatisfactory, and the "copper-lime" precipitation was applied to the glycogen hydrolysates, with satisfactory results, so far as the actual estimation was concerned. Another difficulty, however, arose. We had been accustomed to extract our tissues with 60 % alcohol, to estimate the free sugar and lactic acid in the extract, and to use the extracted tissue for glycogen estimations. Dr P. Eggleton, however, informed us that he had found that glycogen was appreciably soluble in alcohol of less strength than 80 %. This clearly invalidated our glycogen estimations. We take this opportunity of thanking Dr Eggleton for thus generously placing unpublished results at our disposal. By extracting the tissue with absolute alcohol, and estimating glycogen and lactic acid, or with 60 % alcohol, and estimating free carbohydrate and lactic acid, we were able to surmount the difficulty. We were very relieved to find that, although we had been losing at least half of the glycogen in our original experiments, our practice (recommended by Meyerhof) of acidifying the 60 % alcoholic extract had resulted in its subsequent hydrolysis during the rather prolonged process of evaporation, so that it reappeared as "free carbohydrate." Thus,

though our estimations gave an erroneous picture of the distribution of carbohydrate, they presented a reliable one of its total amount—the most important thing from our point of view. Tables II, II*a* and III and III*a* illustrate the point. Needless to say, in our subsequent "free carbohydrate" estimations, we did not acidify the alcoholic extracts.

The technique of a complete experiment was as follows: a rabbit was killed by a blow on the head, the lower part of the body and hind limbs were skinned as quickly as possible and the sciatic nerves dissected out and placed in a dish standing on ice. One to three other animals were treated successively in the same way, the nerves being so arranged that one dish contained the right sciatic nerve of the first, the left of the second, and the right of the third, and so on.

The nerves were well exposed before being handled, and were then held at the distal end with forceps and removed, as far as possible, without being stretched or pulled. One set ("initial") was weighed and at once put into chilled alcohol. The other set was then weighed and placed in a vacuum tube, having a turned-over, hollow stopper containing moist cotton wool (to obviate drying of the nerve). The tube was evacuated and filled with nitrogen[1], evacuation and refilling being repeated three or four times. It was then placed in an incubator at 37° for 1 to 3 hours, or for 1 hour and then left at room temperature over night, or simply left at room temperature. Finally, the tube was cooled, and the nerves were placed in chilled alcohol as already described.

The question arises as to how far these nerves, cooled during dissection, can be regarded as being in a normal condition during the remainder of the experiment. We have tested nerves treated in this manner by their action currents and find that the response remains undiminished for some hours. Also, experiments (to be reported later) have shown that the oxygen consumption of rabbit nerve is not diminished by a preliminary period at 0°; and we believe the findings here reported may therefore be accepted as normal for the resting isolated nerve.

The results of experiments on anaerobic survival of nerve are given in Tables II and II*a*. In Table II, the experiments are those in which the glycogen values are too low, the loss being compensated for by a corresponding increase in the "free carbohydrate." The two values are therefore taken together as "total carbohydrate." In Table II *a* the values for the two carbohydrate reactions are, we believe, correct. It will be seen that the average values for the two tables agree remarkably well. In Table II the lactic acid was estimated by the amyl alcohol method; in Table II *a* by the Schenk technique, except in the case of experiments (1) and (2).

It is plain that lactic acid is produced anaerobically by peripheral nerve at the expense of carbohydrate stored in the tissue. The agreement between the rise in lactic acid and the fall in total carbohydrate is sufficiently good

[1] Purified with red-hot copper filings, and stored over pyrogallol.

Table II. *Rest in nitrogen.*

All values in mg. per 100 g.

	Initial			Final			Differences			
Exp.	Total carbohydrate	Lactic acid	Total	Total carbohydrate	Lactic acid	Total	Lactic acid	Total carbohydrate	Conditions All in nitrogen	Lactic acid method
1	100·6	63·0	163·6	44·1	89·0	133·1	+26·0	−56·5	37°, 1¼ hrs. Room temp. 22 hrs.	[Meyerhof, 1920]
2	83·6	63·7	147·3	68·8	105·4	174·2	+41·7	−14·8	37°, 3 hrs.	,,
3	107·6	101·0	208·6	66·6	134·0	200·6	+33·0	−41·0	37°, 1⅞ hrs. Room temp. 20 hrs.	,,
4	85·5	77·5	165·0	57·1	103·8	160·9	+26·3	−28·4	37°, 2½ hrs.	,,
5	109·6	72·3	181·9	71·3	114·2	185·5	+41·9	−38·3	37°, 1½ hrs.	,,
6	104·8	82·4	187·2	50·6	126·6	177·2	+44·2	−54·2	37°, 1½ hrs.	,,
Av.	98·9	76·6	175·6	59·7	112·1	171·9	+35·5	−39·2		

In Exp. 6, animal was pithed, artificial respiration used, and legs cooled to below 10°, before nerves were dissected.

Friedemann and Kendall [1929] state that they obtain a yield of 95–98 % of lactic acid, using their manganous sulphate reagent, and aeration. In our own experiments we have obtained consistent yields of 91·5 % (without aeration). If the figures in Tables II and II *a* are corrected on this basis, the rise in lactic acid becomes 38·5 mg. per 100 g. and 35·0 mg. per 100 g. respectively, figures very close indeed to the observed fall in total carbohydrate. We have preferred to give the values actually observed, in the tables.

Table II *a*. *Rest in nitrogen.*

All values in mg. per 100 g.

	Initial			Final			Rise or fall				
Exp.	Glycogen	Free sugar	Lactic acid	Glycogen	Free sugar	Lactic acid	Glycogen Fall	Free sugar Fall	Lactic acid Rise	Conditions	Lactic acid method
1	57	—	45	47	—	58	10	—	13	2 hrs. in N_2 at 37°	[Meyerhof, 1920]
2	67	—	87	54	—	116	13	—	30	,,	,,
3	53	—	67	40	—	110	13	—	43	,,	Schenk
4	—	—	68	45	—	101	—	—	33	,,	,,
5	—	37	101	—	17	116	—	20	15	,,	,,
6	—	26	70	—	12	101	—	14	31	,,	,,
7	—	45	76	—	24	119	—	21	43	,,	,,
8	—	61	88	—	23	140	—	38	56	,,	,,
Av.	59	42	75·2	46·5	19	107·5	12·5	23	32·3		
	Total 176·2			Total 173·0							

Average total carbohydrate: Initial 101 Final 65·5

to make it extremely probable that there is no lactic acid precursor concerned other than glycogen and free carbohydrate; of these "free carbohydrate" contributes about twice as much as does glycogen.

In striking contrast to these results are those obtained when the tissue is kept, not in nitrogen, but in oxygen (Tables III and III *a*). In Table III, the glycogen and free carbohydrate are added together and given as "total carbohydrate" since their distribution was not accurately shown. In Table III *a*, the figures give the true relationship between glycogen and "free carbohydrate."

From these two tables and from Table IV, it is clear that the formation of lactic acid, so evident in nitrogen, is entirely suppressed in oxygen, and further, that the oxidative removal of lactic acid does not occur in nerve. To test this point more rigidly, in certain experiments, which appear in Table IV, nerves were kept for varying periods in nitrogen, and were then exposed to oxygen, in the hope that the accumulation of lactic acid might be oxidised away, even if the ordinary "initial" content were not. The results were, however, uniformly negative. The apparent drop in lactic acid in Table III is due to the comparatively small number of experiments, and the random errors of the method. It disappears when a larger group of values is considered, as in Tables III *a* and IV.

Although there is no oxidative removal of lactic acid, there is a very definite fall in carbohydrate, which affects only the "free carbohydrate" fraction (Table III *a*). There is, if anything, a slight rise in glycogen. The contrast between the events which occur in oxygen and in nitrogen are well illustrated by the following figures, which were obtained by dividing the nerves from 6 rabbits each into three portions, which were then distributed to give the best sampling. One set was kept for 1 hour in nitrogen, one for 1 hour in oxygen, and one set was worked up immediately.

Values in mg. per 100 g.

	Initial	Final in N_2	Final in O_2
Total carbohydrate	109·6	71·3	92·6
Lactic acid	72·3	114·2	74·8
Total	181·9	185·5	167·7

We have not been able to gauge successfully the precise time relationships of these events. To construct a curve would necessitate the use of 8 or 10 rabbits, and the time taken in dissection would become unduly long: moreover, it would be necessary, for purposes of sampling, to divide each nerve into four or five pieces, and it is more than likely that the mechanisms concerned would suffer in the process. The tables, however, suggest that the processes taking place in oxygen are more or less complete by the end of the first hour at 37°—the values in Table III are as large as those in Table III *a*, and the same statement applies to those changes which occur in nitrogen.

Table III. *Rest in oxygen.*

Values in mg. per 100 g.

	Initial			Final			Differences				
Exp.	Total carbohydrate	Lactic acid	Total	Total carbohydrate	Lactic acid	Total	Total carbohydrate	Lactic acid	Total	Treatment	Lactic acid method
1	90·5	90·3	180·8	74·1	78·5	152·6	−16·4	−11·8	−28·2	37°, 3¼ hrs.	[Meyerhof, 1920]
2	102·0	78·0	180·0	85·5	74·0	159·5	−16·5	− 4·0	−20·5	37°, 1 hr.	„
3	117·5	96·0	213·5	87·6	78·0	165·6	−29·0	−18·0	−47·9	37°, 1 hr.	„
4	109·6	72·3	181·9	92·6	74·8	167·4	−17·0	+ 2·5	−14·5	37°, 1 hr.	„
5*	100·9	84·7	185·6	63·2	90·5	153·7	−37·7	+ 5·8	−31·9	37°, 1 hr.	„
Av.	104·1	84·2	188·3	80·6	79·1	159·7	−23·5	− 5·1	−28·6		

* Nerves from 5 decerebrated cats, 3·5 g. of tissue available for each estimation.

Table III *a*. *Rest in oxygen.*

All values in mg. per 100 g.

	Initial			Final			Differences				
Exp.	Glycogen	Free carbohydrate	Lactic acid	Glycogen	Free carbohydrate	Lactic acid	Glycogen	Free carbohydrate	Lactic acid	Treatment	Lactic acid method
1	—	29	98	—	19	91	—	−10	− 7	2 hrs. at 37° in O_2	Schenk
2	—	45	108	—	25	109	—	−20	+ 1	,,	,,
3	—	72	104	—	24	99	—	−48	− 5	,,	,,
4	—	45	113	—	21	98	—	−24	−15	,,	,,
5	50	—	65	54	—	77	+4	—	+12	,,	,,
6	55	—	57	57	—	62	+2	—	+ 5	,,	,,
7	55	—	63	57	—	65	+2	—	+ 2	,,	,,
Av.	53·3	47·7	86·8	56	22·2	85	+2·6	−25·5	− 1·1		
	Total 187·8			Total 163·2							

Table IV.

Lactic acid (mg. per 100 g. fresh nerve)		
Initial (A)	Final (B)	Treatment
104·2	95·1	Both 1½ hrs. at 37° in N_2 (B) Next 2 hrs. in air at 37°
80·1	102·1	Both, 1 hr. at 37° in N_2 (B) Next 1½ hrs. at 37° in O_2
90·3	78·5	(A) "Resting" (B) 3½ hrs. in O_2 at 37°
36·9	37·7	(A) "Resting" (B) 3 hrs. in O_2 at 37°
87·9	104·4	(A) "Resting" (B) 1 hr. in O_2 at 37°
78·0	74·0	(A) "Resting" (B) 1 hr. in O_2 at 37°
96·0	78·0	(A) "Resting" (B) 1 hr. in O_2 at 37°
72·3	74·8	(A) "Resting" (B) 1 hr. in O_2 at 37°
Average 77·3	78·5	

The question naturally arises as to the precise nature of the "free carbohydrate" estimated. Since the fall in free carbohydrate agrees well with the rise in lactic acid, it seems likely that, in so far as it gives rise to lactic acid, it is really hexose. Clearly, however, the possibility that it is hexose-phosphate must be considered. The reducing power of a molecule of hexose-monophosphate is about 2/3 of that of a molecule of glucose, and that of hexosediphosphate about 1/3[1]. If, therefore, either mono- or diphosphate breaks down to give rise to lactic acid and free phosphoric acid, more lactic acid should appear than can be accounted for by the apparent fall in "free carbohydrate," and there should be a loss of combined phosphate from the free carbohydrate fraction. If, however, as suggested by Meyerhof [1926] two molecules of monophosphate give rise to two molecules of lactic acid and one of diphosphate, then (*a*) one molecule of glucose will appear to have given

[1] Eggleton: personal communication.

rise to an equivalent weight of lactic acid, since the reducing value of 2 mols. of monophosphate = 4/3 mol. of glucose and the reducing value of 1 mol. of diphosphate = 1/3 mol. of glucose (4/3 − 1/3 = 1); and (*b*) there will be no change in combined phosphate.

Table V.

Total P in mg. per 100 g. of fresh nerve.

Exp.	Initial	Final
1	9·6	9·9
2	7·7	10·3
3	7·2	8·4

Table V gives the values obtained in three experiments, where the total phosphate was estimated in the carbohydrate fraction before and after incubation for 2 hours at 37° in nitrogen. In all three experiments the material obtained by the Bissinger precipitation was incinerated with H_2SO_4 and "perhydrol." The phosphorus was estimated in the first two experiments by the Briggs method, in the third by Pregl's micro-method, for the performance of which we have to thank Mr A. Colwell. So far from there being a loss of organic phosphorus, there is actually a rise. In the absence of further data, it is hardly profitable to discuss the possible origin of this increase, but we feel that we are justified in concluding that the lactic acid arises either directly from glucose, or, if from hexosephosphate, then along lines such as those indicated by Meyerhof, and not by any process involving loss of organically combined phosphate.

It is clear that, in any case, the "free sugar" fraction must contain substances other than glucose, since (1) organically combined phosphorus is present, (2) a positive orcinol test (for pentose) can be obtained, and (3) it is capable of yielding lactic acid only to a limited extent (Table II *a*), though in frog nerve [Gerard and Meyerhof, 1927] lactic acid seems to be formed as long as glucose is present. It is of some interest to compare the data presented in this paper with those available from other sources. Calculating from the figures obtained for frog nerve at 15° [Gerard and Meyerhof, 1927], and assuming a temperature coefficient of 2·5, we obtain a value for lactic acid formation by frog nerve at 37° of 35 mg. per hour. Rabbit nerve forms about 36 mg. in two hours, and there is reason to suppose that most of that formation is complete within the first hour.

Gerard and Gruskin (unpublished) find an oxygen consumption for rabbit nerve at 37° of about 280 mm.^{3} per g. nerve per hour, and this has recently been confirmed by Dr M. Sherif (unpublished). The oxygen equivalent of the sugar disappearing during rest in oxygen, 0·23 mg. per g. per hour (supposing for the moment such sugar to be burned) is 192 mm.^{3} per g. per hour, representing about 68 % of the total oxygen uptake of the nerve at rest in oxygen. Comparing the results here obtained for nerve with those for brain, it is clear that the rate of metabolism in the two tissues is widely different, apart from

the fact that brain oxidises lactic acid freely, while peripheral nerve does not do so at all. Values for oxygen consumption of brain admittedly present great difficulty. Most of those available refer to anaesthetised brain, and are therefore only of very limited value for comparison; the only determinations on unanaesthetised brain of which we are aware are those of Alexander and Cserna [1912, 1913]; they are so enormous that they can hardly be accepted without confirmation. For anaesthetised dog's brain Alexander and Cserna give 144–36 mm.^{3} per g. per min.; Hou [1926], and Hou and Sigiura [1926] 60 mm.^{3}. Schmidt's [1928] figures approximate to 75 mm.^{3} for amytal anaesthesia. The figure for nerve is about 4·6 mm.^{3} per g. per min., and the nerve is not anaesthetised; the contrast is sufficiently striking. Similarly, the rate of lactic acid formation is much greater in brain than in nerve [Holmes and Holmes, 1925; McGinty and Gesell, 1925].

Results on stimulated nerve, obtained by the two authors independently, and using rather different technical procedures, indicate that the carbohydrate changes discussed in this paper bear no part whatever in the extra metabolism of stimulation. In this connection, it is noteworthy that the carbohydrate disappearance in oxygen appears to be complete in two hours; but a nerve, kept in oxygen, will continue to give an electric response to stimulation for at least 4 or 5 hours.

Summary.

1. A modification of the Van Slyke-Bissinger method for separation of carbohydrates from tissue extracts is described.

2. The content of glycogen, "free sugar" and lactic acid of rabbit nerves has been studied in the fresh tissue and after a period of rest in oxygen or nitrogen. We have shown that (*a*) in nitrogen there is rise in lactic acid, and a corresponding fall in carbohydrate; (*b*) in oxygen there is no disappearance of lactic acid, but a definite fall in the carbohydrates.

3. About 60 % of the resting metabolism of rabbit nerve may be maintained by the oxidation of these carbohydrates. The rate of lactic acid formation under asphyxial conditions is comparable to that of frog nerve and much less than that of rabbit brain. No oxidation or resynthesis of lactic acid occurs on admitting oxygen to an asphyxiated nerve.

4. The formation of lactic acid, even if concerned with hexosephosphate, does not involve breakdown of mono- or di-phosphate with liberation of free phosphate.

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LXXXV. CONTRIBUTIONS TO THE STUDY OF BRAIN METABOLISM.

V. RÔLE OF PHOSPHATES IN LACTIC ACID PRODUCTION.

By CHARLES AMOS ASHFORD and ERIC GORDON HOLMES.

From the Biochemical and Pharmacological Laboratories, Cambridge.

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We have endeavoured to investigate the part played by inorganic phosphate in the production of lactic acid from carbohydrate by surviving brain tissues. Winterstein and Hecker [1923] have studied the phosphorus metabolism of the isolated spinal cord of the frog, and Gerard and Wallen [1929] have investigated that of peripheral nerve, both amphibian and mammalian. While, however, previous work had demonstrated the ready production of lactic acid by brain tissues, both *in vitro* [Warburg, Posener and Negelein, 1924] and *in vivo* [Holmes and Holmes, 1925, 1926, 1927], we believe that no serious attempt has previously been made to study the part played by phosphate in the process. It was natural to suppose that it would be a prominent one and would follow, more or less closely, the lines already established by Harden [1923], Embden [1926], Meyerhof [1926, 1] and many other workers, for the metabolism of yeast and of striped muscle.

This supposition has turned out to be erroneous; it appears that brain tissue possesses two mechanisms for the production of lactic acid, and that of these the one that has the greater quantitative significance is independent of the presence of phosphate.

Methods.

Rabbits were used for all experiments. They were killed by a sharp blow on the back of the neck, the brains excised as quickly as possible, and chilled by being placed in a glass basin surrounded by ice. When cold, they were freed from membranes and adherent blood-clot, chopped, and sampled. Portions weighing 0·5–2·0 g. were weighed out as rapidly as possible. For "resting" or "initial" values, the tissue was at once transferred to chilled 20 % trichloroacetic acid (B.D.H. "A.R."). It was ground with sand, after standing 1–2 hours or sometimes overnight, and filtered through a small filter-paper into a 50 cc. volumetric flask. After three further extractions with 10 % trichloroacetic acid, the whole process being spread over 1½ hours,

the contents of the flask were made up to 50 cc. with water. Phosphates were estimated by the Briggs [1924] method. "Phosphagen" therefore appeared as inorganic phosphate. For the estimation of lactic acid, 30 cc. of the filtrate were taken: the Van Slyke copper-lime precipitation was carried out, and the lactic acid estimated by the method of Friedemann, Cotonio and Shaffer [1927]. Blank distillations were regularly performed, the figures obtained varying between 0·1 and 0·3 cc. of 0·005 *N* iodine. Our yields against zinc lactate were 93 % [see Friedemann and Kendall, 1929].

To observe the effect of various substances on lactic acid formation and phosphate liberation, the chopped tissue was suspended either in Ringer's or buffer solution contained in a boiling-tube. This was closed with a rubber stopper, fitted with glass exit tube, rubber tubing, a clip, and sealed with paraffin wax. The tubes were immersed in a water thermostat at 37°. They were evacuated, and sometimes filled with nitrogen, but the presence or absence of nitrogen did not affect the result.

In many of the experiments quoted in this paper we have suspended the tissue in borate buffer. We have also carried out a number of experiments in which Ringer's solution was used as the suspension medium, and the results have been quite similar to those quoted. It also appears to be immaterial (so far as liberation of phosphate is concerned) whether strictly anaerobic conditions are maintained (as in these experiments) or whether the tissue is merely incubated in an open beaker. Both the maintenance of strictly anaerobic conditions and the use of borate buffer are, however, of importance with regard to experiments described later, and we have therefore, for the sake of uniformity and simplicity, quoted for the most part experiments where similar conditions prevailed.

We deliberately chose first to investigate the possibility of hexosephosphate formation from glucose rather than from glycogen. We were primarily interested in its rôle in connection with the production of lactic acid, and, while brain tissue produces lactic acid very freely from glucose, its production from glycogen is much less impressive.

Table I shows the liberation of free phosphate by chopped brain tissue at 37° under anaerobic conditions, and the effect of glucose on this process. The liberation of phosphate is a well-marked phenomenon, and it is inhibited, to a slight, but quite definite extent, by the presence of glucose. This fact might be interpreted as meaning that there is a certain formation of hexosephosphate from glucose by chopped brain tissue. The inhibition of phosphate liberation amounts to about 4 mg. per 100 g., and this would correspond to the formation of 24 mg. hexosemonophosphate per 100 g. tissue, in the space of 2 hours. Controls using boiled brain show no increase of lactic acid on incubation with glucose. Inorganic phosphate, however, increases—5 mg. per 100 g. in one case and 9 mg. per 100 g. in another. In each case the weighed sample was dropped into boiling buffer solution and subsequently placed on a boiling water-bath for some minutes.

Table I.

All values in mg. phosphorus per 100 g. fresh tissue. Anaerobic.

	No glucose					Glucose 0·4 %				
Exp.	Initial	Final	Difference	Time of incubation (hrs.)	Incubated in	Initial	Final	Difference	Time of incubation (hrs.)	Incubated in
1	48·0	62·0	14·0	2	Ringer	34·6	47·8	13·2	2	Ringer
2	41·6	55·0	13·4	2	,,	37·3	49·7	12·4	2	,,
3	43·0	67·0	24·0	2·25	,,	39·2	56·2	17·0	2	Borate buffer
4	40·8	60·6	19·8	2	Borate buffer	48·9	59·0	10·1	2	p_H 8·2
5	41·2	64·8	23·6	2	p_H 8·63	38·3	59·2	20·9	2	,,
	39·0	64·0	25·0	2	p_H 9·03	40·8	55·8	15·0	2	Borate buffer
						41·2	59·4	18·2	2	p_H 8·63
	31·0	63·4	32·4	3	p_H 8·63	31·0	54·6	23·6	3	,,
	44·2	68·5	24·3	3	,,	44·2	64·9	20·7	3	,,
						40·0	56·4	16·4	2	,,
	31·0	40·3	9·3	Boiled	,,					
	44·2	49·2	5·0	,,	,,					

The possibility of demonstrating hexosephosphate synthesis in this way depends, of course, upon the assumption that the breakdown of hexosephosphate is a slower process than its synthesis. Thus, Meyerhof [1926, 1, 2], using the muscle enzyme and starch or glycogen, was able to demonstrate a very definite disappearance of free phosphate during the early stages of incubation. Our failure to observe a more marked disappearance might, therefore, have been due to the fact that, after the lapse of 2 hours, the process of breakdown had outstripped that of synthesis. Table II shows two experiments where the liberation of free phosphate, both in the presence and absence of glucose, was followed at short intervals of time. There is, however, no evidence of esterification of phosphate. From these experiments, therefore, we have obtained, at best, evidence that may be interpreted as indicating hexosephosphate synthesis to only a very limited extent.

Table II. *Time of liberation of inorganic phosphate by brain tissue, with and without glucose.*

Mg. phosphorus per 100 g. fresh tissue. Tissue suspended in Ringer's solution.

	Time (mins.)	With glucose	Without glucose
1.	0	44·7	44·7
	10	53·4	—
	40	57·3	56·4
	80	70·2	72·8
2.	0	45·0	45·0
	10	49·1	46·7
	20	51·2	51·1
	40	56·8	48·6

Our next step was, therefore, to examine the effect of adding sodium fluoride to the preparations. Embden and Hayman [1924] were in this way able to demonstrate the synthesis of hexosephosphate by muscle from added

glycogen, and we hoped that we should be able in a similar manner to inhibit the breakdown of any hexose ester that might be formed from glucose.

We were at once struck by the very feeble inhibiting effect exerted, even by high concentrations of fluoride, on the liberation of free phosphate. In the presence of concentrations of fluoride, such as 0·05 *M*, the effect was only occasionally observed. Even with the highest concentrations, inhibition was never complete, nor was it constant from experiment to experiment.

We next observed the effect of different fluoride concentrations both on lactic acid production and on phosphate liberation. The results of such an experiment are shown in Table III. It is obvious that the two phenomena bear no quantitative relationship to each other; inhibition of lactic acid production is marked, and at concentrations of fluoride as low as 0·002 *M* it amounts to 64 %. The data in Table IV tell the same story; in addition, another point becomes clear. If the inhibition of lactic acid production depends on the formation of hexosephosphate, and its stabilisation by fluoride, then for every two molecules of lactic acid, the production of which is inhibited, a definite amount of phosphorus should change from inorganic to organic combination. This amount should be either one or two atoms, according as the mono- or di-phosphate is the substance involved. The atomic weight of phosphorus being 31, and the molecular weight of lactic acid 90, the amount of phosphorus disappearing—or, at least, prevented from appearing as inorganic phosphate—would accordingly be either one-sixth or one-third of the amount of lactic acid inhibited. Now it is perfectly clear that in no case does the inhibition of phosphate approach the figure required to account for the observed inhibition of lactic acid, even on the basis of monophosphate formation. This seems to make the conclusion inevitable that, in the case of lactic acid formation by brain tissue from glucose, the inhibition by fluoride must depend on some mechanism other than the stabilisation of hexosephosphate, which is that usually postulated to account for the inhibition occurring in other tissues.

Table III. *Effect of varying concentrations of sodium fluoride on inorganic phosphate and lactic acid production of chopped brain incubated anaerobically for* 2 *hrs. with* 0·4 % *glucose in borate buffer* p_H 8·2.

	Mg. of phosphorus and lactic acid per 100 g. fresh tissue.			
	Phosphorus	% inhibition	Lactic acid	% inhibition
Initial value	39·2	—	157	—
NaF 0·1 *M*	53·9	13	185	88
„ 0·02 *M*	56·2	0	191	85
„ 0·01 *M*	58·6	0	202	81
„ 0·005 *M*	59·0	0	222	72
„ 0·002 *M*	59·6	0	240	64
No NaF	56·2	—	391	—

So far, the only piece of evidence that we could bring forward in favour of hexosephosphate formation was the slight decrease in the amount of phosphate liberated when brain was incubated in the presence of glucose.

Our experience with fluoride certainly told heavily in the opposite direction. We tried to attack the problem, therefore, from another side, and endeavoured to demonstrate the formation of lactic acid by brain tissue in circumstances in which free phosphate had been eliminated as an active participant. We first tried, unsuccessfully, to render brain tissue free from phosphate by repeated washings with warm Ringer's solution. We next attempted to immobilise the free phosphate by addition of $CaCl_2$ at alkaline reaction. Two samples of chopped brain tissue were suspended in 4 cc. borate buffer, p_H 8·15, containing 1 cc. of 1·2 % $CaCl_2$ per g. tissue. They were allowed to stand for 30 minutes at room temperature. Glucose was then added to make the final concentration 0·4 %, and both samples were incubated for 2 hours, anaerobically. They were then removed from the incubator, and the lactic acid formation in one of them determined by the usual method. The other was centrifuged, the supernatant fluid, which was still turbid, was withdrawn, and the precipitate ground with sand and washed at the centrifuge with a little borate buffer; the washings were added to the fluid, and acid ammonium molybdate added in quantity slightly greater than that employed in the Briggs estimation. The fluid was rapidly filtered, and to the clear filtrate the remaining Briggs's reagents were added in proper proportions. In several such experiments either there was no development of colour whatever, or a very faint tinge of green appeared on long standing.

Table IV. *Effect of fluoride on liberation of phosphate by brain tissue.*

Mg. of phosphorus or lactic acid per 100 g. fresh tissue, incubated at 37° anaerobically.

		Phosphorus						Lactic acid			
Incubation time (hrs.)	Amount glucose %	Initial	Final, no NaF	Final, NaF present	Inhib.	Concentration NaF	Suspension medium	Initial	Final, no NaF	Final, NaF present	Inhib.
2	Nil	41·6	55·0	52·4	2·6	0·1 *M*	Borate buffer p_H 8·2	—	—	—	—
3	0·4	41·7	59·2	49·9	9·3	0·5 *M*	Ringer	124	232	125	108
2	0·4	37·8	49·7	47·4	2·3	0·1 *M*	,,	131	332	133	199
2	0·4	34·6	47·8	39·7	8·1	0·1 *M*	,,	162	356	161	195
2	0·25	40·8	55·8	55·0	0·8	0·1 *M*	Borate p_H 8·63	132	430	143	287
2½	0·25	41·2	59·4	57·9	1·5	0·1 *M*	,,	136	331	144	187
3	0·25	31·0	54·6	45·8	8·8	0·1 *M*	,,	143	470	165	305
3	0·25	44·2	64·9	61·8	3·1	0·1 *M*	,,	129	330	134	196
2	0·25	40·0	56·4	53·1	3·3	0·1 *M*	,,	138	397	153	244

This was accepted as evidence that the preparation was free from soluble phosphate. The tissue was ground with sand, so there can be no question of phosphate being precipitated from the fluid medium, but remaining inside the cells, in soluble form; to assure ourselves on this point, in several experiments the tissue was ground up with sand before being incubated with glucose.

It will be seen from Table V that these preparations form lactic acid in amounts quite comparable to those formed in other circumstances; in other

words, lactic acid is still formed by brain tissue even when there is no inorganic phosphate present in soluble form. This seems to make it quite certain that there can be no intermediate formation of hexosephosphate as a step in the process. It is of interest that borates do not interfere with the formation of lactic acid from glucose by brain tissue, since it has been stated that they do prevent lactic acid production in muscle [Chrzaszezewski and Mozolowski, 1928].

Table V. *Lactic acid production by brain tissue from glucose, in medium rendered free from phosphate, in 2 hours' incubation at 37° anaerobically.*

Mg. lactic acid per 100 g. fresh tissue.

Procedure	Final lactic acid content	p_H of buffer
Tissue chopped	283	8·15
"	233	8·15
Tissue chopped and ground with sand	240	8·15
" " "	235	8·15
" " "	218	8·15
Tissue chopped	260	8·89
Average initial value	130	

In an endeavour to clinch the point, we performed experiments to see whether the addition of extra phosphate to preparations previously rendered phosphate-free by the addition of calcium, would have any effect on the velocity of lactic acid formation. The procedure was as follows. Four samples consisting of equal weights of chopped brain tissue were put up in borate buffer at various p_H containing calcium (1 cc. 1·2 % $CaCl_2$ per g. tissue) as before. They were allowed to stand, sometimes at room temperature, sometimes in ice, for 30 minutes. To two of them were then added amounts of Na_2HPO_4 solution calculated to precipitate all the calcium (1 cc. 2·6 % Na_2HPO_4, $12H_2O$ per g. tissue) and to leave a definite excess of phosphate. The addition of the phosphate and precipitation of calcium naturally caused a shift in p_H towards the acid side. The two samples were therefore balanced against each other on a hydrogen electrode apparatus and the p_H of the sample to which phosphate had been added was brought back to its original value by the addition of alkali; the dilution which resulted being compensated by the addition of distilled water to the other sample. Glucose was then added to bring the concentration to 0·4 %, and all four samples were incubated anaerobically, two for 1 hour, the other two for 2 hours.

The results shown in Table VI are not completely regular, but they seem to show that the presence of phosphate causes only a very slight increase in the rate of lactic acid formation either in the first or second hour. The probable reason for this small effect of phosphate will be discussed immediately; meanwhile it appears that, so far as glucose is concerned, the part played by phosphate in lactic acid formation is negligible.

While there seems to be no doubt that the glucose-lactic acid mechanism is independent of the intervention of phosphate, we wished to explore the possibility of the existence of a separate mechanism for lactic acid formation,

involving glycogen and hexosephosphate, and going forward on a much smaller scale.

Table VI. *Velocity of lactic acid formation from glucose, (a) in the absence, (b) in the presence of phosphate. Incubated anaerobically.* p_H *of borate buffer as indicated.*

Mg. lactic acid per 100 g. fresh tissue.

		Phosphate absent					Phosphate present			
Exp.	p_H	Initial	End of 1st hr.	End of 2nd hr.	Formed in 1st hr.	Formed in 2nd hr.	End of 1st hr.	End of 2nd hr.	Formed in 1st hr.	Formed in 2nd hr.
—	8·73	137	157	190	20	33	163	193	26	30
—	8·89	126	175	256	54	81	179	270	58	91
70	8·83	140	156	228	16	72	181	255	41	74
71	8·83	124	203	245	79	42	213	246	89	33
72	8·83	126	182	215	56	33	188	232	62	44
75	9·03	128	164	189	36	25	161	221	33	60
—	9·03	138	173	201	35	28	185	204	47	19
Average		131	173	218	42	45	180·5	232	50·5	51·5

It is notoriously difficult to demonstrate the degradation of added glycogen by surviving tissues (as opposed to tissue extracts) even in the case of such organs as muscle, which are known to produce lactic acid freely from glycogen stored in the cells themselves. If chopped brain is incubated in buffer or Ringer's solution containing 0·4 % glycogen, very little more lactic acid is produced than by the brain tissue alone. This observation is in accordance with that of Loebel [1925]. Thus, the average initial value for lactic acid in brain tissue was found to be 112 mg. per 100 g., which rose to 136 mg. on incubation without glycogen, Meyerhof's method [1920] being used for lactic acid estimation. The rise in this case was 22 mg. per 100 g. Table VII shows that when glycogen is added in concentrations as high as 0·4 %, the increase is only 30 mg. per 100 g. The experiments (see Table VIII) on the inhibition of phosphate liberation by sodium fluoride when brain is incubated with glycogen show that the phosphate which fails to appear (a much larger quantity than is the case when glucose is added instead of glycogen (Table IV)) is more than sufficient to account for the observed inhibition of lactic acid formation by the stabilisation of hexosephosphate.

Table VII. *Production of lactic acid by brain tissue incubated with 0·4 % glycogen.*

Mg. lactic acid per 100 g. fresh tissue.

	Initial	Final	Medium
	122	162	Ringer
	137	193	,,
	138	146	,,
	130	143	Borate p_H 8·63
	126	142	,, 8·63
	125	144	,, 8·63
	125	183	,, 8·63
Average	129	159	

Table VIII. *Brain tissue incubated anaerobically with* 0·4 % *glycogen in Ringer's solution or borate buffer* p_H 8·63 *for* 2 *hours.*

All values in mg. per 100 g. fresh tissue.

Phosphorus (inorganic)				Lactic acid				
Initial	Incubated, no fluoride	Incubated, with 0·1 *M* NaF	Inhibition of P	Initial	Incubated, no fluoride	Incubated, plus 0·1 *M* NaF	Inhibition of lactic acid	Inhibition of P required to account for formation of diphosphate
37·8	50·7	38·5	12·2	122	162	131	31	10·1
45·0	66·4*	57·8	8·6	120	141	121	20	6·6
40·7	69·0†	57·5	11·5	126	163	141	22	7·3
40·7	68·5	57·5	11·0	—	—	—	—	—
39·0	62·5‡	51·0	11·5	126	162	134	28	9·3
39·0	61·0	50·8	10·2	126	172	129	43	14·0

* Inc. without glycogen 70·0
† " 75·7
" +NaF 68·3
‡ " 65·5
" +NaF 57·0

Table IX.

Lactic acid, mg. per 100 g. fresh tissue.

	Initial	Incubated with glycogen	Incubated with glycogen and $CaCl_2$ ("phosphate-free")	Incubated with glycogen, $CaCl_2$ and phosphate (phosphate)	p_H of buffer (borate)
	126	142	136	162	8·63
	125	144	129	144	8·63
	125	183	136	163	8·63
	130	143	127	134	8·63
Average	126	153	132	151	

Finally, we determined the effect of incubating brain tissue with glycogen, in the absence of soluble inorganic phosphate, and after the addition of Na_2HPO_4, just as was done in the case of glucose. The results, presented in Table IX show that, when soluble inorganic phosphate is "immobilised" by calcium, the production of lactic acid is completely inhibited, while the addition of excess of phosphate restores the power of lactic acid formation almost to its original condition. This offers a sufficiently striking contrast to the state of affairs which exists in the parallel experiments when glucose, and not glycogen, is the substrate, and it appears to offer an explanation of the slightly greater velocity of lactic acid formation in the presence of glucose when phosphate is replaced, for the tissue itself must be presumed to contain a certain amount of glycogen or hexosephosphate, or both, and the conversion of this into lactic acid would then be checked by the presence of calcium, and would proceed normally in the experiments in which phosphate was restored. We have on one occasion observed a production of lactic acid from sodium

hexosediphosphate. Under the same conditions a rise of 127 mg. per 100 g. was observed with glucose at the same hexose concentration, while the diphosphate gave a rise of 55 mg. per 100 g. (p_H 8·85 at 37° for 2 hours, anaerobically).

Lactic acid, therefore, can be produced by brain tissue in two distinct ways: from glucose, by some mechanism which does not involve the intervention of phosphate, and from glycogen, by a mechanism in which phosphate plays an essential part, and which is, presumably, akin to that already made familiar by studies on muscle.

Both processes are inhibited by sodium fluoride, and the production of lactic acid from glucose is evidently a mechanism which is very sensitive to the action of this substance. If, however, our findings are correct, this inhibition can no longer be explained on the lines at present current, namely the formation and stabilisation of a hexose ester, since no hexose ester is formed.

We have no certain knowledge of the origin or meaning of the phosphate production which occurs. Holmes and Holmes [1926] have previously shown that both a pentose and hypoxanthine can be demonstrated in brain tissue, and it seems clear that nucleotides play a part in the metabolism of nerve tissue, as they do in that of muscle [Embden, 1926; Parnas, 1929]. Gerard and Wallen [1929] have shown that pyrophosphates are present in nerve, while the possibility of phosphates arising from some of the many phosphorus-containing lipins of brain is almost unexplored. There is therefore no need, at present, to assume that phosphate liberation has necessarily any connection with carbohydrate metabolism.

The direct evidence that we can bring to bear on the question of the origin of the phosphate which is liberated is chiefly negative in character, and falls under four headings.

(1) The amount of pyrophosphate in brain tissue, as estimated by the method of Lohmann [1928] is small, and Table X shows that it can account for only a trifling amount of the rise in orthophosphate.

(2) A single experiment was performed with rabbit gut phosphatase prepared by the method of Kay [1928]. A preparation which was very active towards hexosediphosphate failed to cause any rise in inorganic phosphate when incubated with brain tissue.

Table X.

Pyrophosphate-phosphorus of brain, mg. P per 100 g. fresh tissue.

		Before hydrolysis (HCl)	After hydrolysis	Difference (pyrophosphate) P
Resting		47·5	53·0	5·5
Incubated	Glucose	54·4	58·4	4·0
3 hrs. p_H 8·85	No glucose	58·2	61·2	3·0
Ditto	Glucose	63·5	65·3	1·8
for 18 hrs.	No glucose	92·5	93·2	0·8

(3) Estimations of organic, acid-soluble phosphorus by incineration with H_2SO_4 and "perhydrol" show a change of the order, and in the direction, which would be anticipated from the small decrease in the amount of inorganic phosphorus liberated in the presence of fluoride as compared with that liberated in its absence both with, and without, glucose.

(4) Some experiments were performed on tissues which had been extracted with (*a*) acetone only, and (*b*) with acetone followed by ether. The ether was changed once a day for 14 days. These preparations have entirely lost their power of forming lactic acid; but Table XI shows that they had by no means lost their power of liberating inorganic phosphate. It will be seen from the table that the additional treatment with ether lowers both the "initial" and incubated values by about 20 %.

Table XI.

Mg. inorganic P per 100 g. tissue (wet).

In results it is assumed that 1 g. wet tissue = 0·2 g. dry.

Exp.		Acetone-dried	Acetone-ether-dried
1	Initial	58·2	47·5
2		65	—
3		62	61
	Incubated:		
1	2·5 hrs. at 37° with 2·5 % glucose, p_H 8·89	70	62
2	Ditto, p_H 8·43	82 with glucose 82 without glucose	65·5 with glucose 68·1 without glucose
3	3 hrs., p_H 8·43	75 without glucose 77 „ +0·1 *M* NaF 79·5 + glucose 74·5 + „ +0·1 *M* NaF	66 without glucose

Discussion.

Our experiments lead us to believe that brain contains two mechanisms for the production of lactic acid, one from glycogen, requiring the presence of phosphate, and therefore presumably following the same lines as in muscle, *i.e.* by way of hexosephosphate, and the other from glucose which is quantitatively more important, and does not require the presence of inorganic phosphate. In this connection it is of interest that Stiven [1929] has observed a much smaller degree of ester formation by muscle extract during lactic acid formation in the presence of glucose, than in the cases when glycogen was acting as a substrate. Case [1929] reports that muscle extract + brain suspension will produce lactic acid from glucose and that this process uses phosphate, in that inorganic phosphate disappears in the presence of fluoride. In this process it appears, therefore, that the glucose molecule undergoes some change prior to the hexosephosphate stage. The brain activator in some way renders the molecule accessible to the muscle enzymes, which are then capable of behaving towards it as they do towards glycogen. In contrast to this, we have shown that in the production of lactic acid from glucose by

brain alone, a hexosephosphate stage does not occur. In that case the glucose is broken down presumably after the intervention of the activator, through stages of which we have no knowledge at present, but not involving a hexosephosphate. Of the nature of the "activated" glucose, or as to how the activation is brought about, we are as yet ignorant.

SUMMARY.

1. Inorganic phosphate is liberated from brain tissue both anaerobically and aerobically, and in the presence as well as in the absence of glucose. No evidence of hexosephosphate synthesis has been found at any stage in the process of formation of lactic acid, although the tissue is capable to a small extent of performing this synthesis.

2. Both phosphate liberation and lactic acid production from glucose by brain tissue are inhibited by sodium fluoride, but, whilst the former is affected only by a high fluoride concentration, the latter is sensitive to very high dilutions of the salt. No quantitative relationship can be traced between the amounts of phosphate and lactic acid which are prevented from appearing by fluoride.

3. Lactic acid is freely formed from glucose, even when all available phosphate is immobilised. The velocity of lactic acid formation from glucose is not increased by the replacement of phosphate.

4. Much less lactic acid is formed from glycogen than from glucose; the process is inhibited by fluoride and by immobilising phosphate. It can be restored by replacing phosphate.

5. It is concluded that brain tissue possesses two mechanisms of lactic acid formation: one, involving glucose, is quantitatively the more important, and is independent of phosphate; the other is much smaller, involves glycogen, and depends on the availability of phosphate.

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LXXXVI. THE PRODUCTION OF AMMONIA BY SURVIVING KIDNEY TISSUE.

PRELIMINARY PAPER.

By ANTOINETTE PATEY

(*Francis Maitland Balfour Student of Newnham College.*)

AND BARBARA ELIZABETH HOLMES.

From the Biochemical Laboratory, Cambridge.

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SINCE the publication of the work of Nash and Benedict [1921] it has been realised that the production of ammonia for the neutralisation of acid glomerular filtrates is one of the functions of normal kidney tissue. The precursor of this ammonia is not known, but it is certainly not urea. Failure of the ammonia-producing mechanism occurs in certain types of nephritis, and in the urine of such cases the ratio of acid to ammonia is very much higher than in normal urine [Henderson and Palmer, 1915].

The production of ammonia by excised kidney tissue, however, has so far received but little attention, and most of the work that has been done has been concerned with the autolytic production of ammonia, in experiments extending over several days.

It has been shown for other tissues [Warburg, Posener and Negelein, 1924; Meyerhof, Lohmann and Meyer, 1925; Loebel, 1925] that the presence of oxygen increases the rate of ammonia production, but in the absence of oxygen the production still continues to some extent. The effect of aerobic and anaerobic conditions upon the production of ammonia by kidney tissue was therefore first investigated.

Rat (or occasionally rabbit) kidneys were removed as soon as possible after death, chopped finely, washed twice in Ringer's solution, and then suspended in Ringer's solution and aerated with air for about 2 hours. More constant and satisfactory results were obtained after addition of glycine when the tissue had been aerated previously, and it was for this reason that preliminary aeration was carried out in all the experiments described in this paper. For estimating the anaerobic ammonia production the re-washed tissue, after filtration, was weighed (0·4 g. was usually taken) and tipped into a boiling-tube containing 1 cc. 0·1 *M* potassium phosphate (p_H 7·2) and 2 cc. Ringer's solution, or 1 cc. Ringer's solution and 1 cc. of substrate dissolved in Ringer's solution. The boiling-tube was fitted with a rubber stopper and an

outlet tube for evacuation, and small tubes containing alkaline hydrosulphite were suspended inside the boiling-tube to absorb any traces of oxygen. The rubber stopper was waxed thoroughly and the tube evacuated and incubated in the hot room at 37° for 3 hours.

The aerobic ammonia production was estimated after the tissue had been shaken in a Barcroft differential manometer at 37° for 3 hours, the amount of tissue taken and the media employed being similar to those described for the anaerobiosis experiments.

At the end of 3 hours 1 cc. of metaphosphoric acid (or 1 cc. 2 *N*/3 sulphuric acid and 1 cc. sodium tungstate solution) was added to the contents of the anaerobic tubes or Barcroft cups to precipitate proteins, and left overnight. The next day the contents of the tubes or cups were ground with further addition of metaphosphoric acid, and were filtered with repeated washing of the precipitates. The ammonia was estimated on a portion of the filtrate by Holmes and Watchorn's [1927] modification of Stanford's [1923] method.

The amounts of ammonia obtained were ample for estimation by this method, which was known to be a reliable one. All solutions used were free from ammonia.

Table I. *Ammonia production by kidney tissue anaerobically and aerobically.*

Values in mg. NH_3-N

Resting	Anaerobic	Aerobic
0·01	0·033	—
0·01	0·024	—
0·01	0·025	—
0·0069	0·038	0·115
0·0258	—	0·091
—	0·034	0·093
—	0·045	0·077
—	0·028	0·120

Duration of experiments—3 hours.

We always obtained a small production of ammonia anaerobically, and a larger production aerobically, as shown by the examples given in Table I. In unwashed and non-aerated tissue the aerobic production was larger than in the examples shown, and it is quite possible that the anaerobic production would also be larger under these circumstances.

When small amounts of glycine were added to the medium (final concentration 0·02 *M*) the ammonia production was much increased under aerobic conditions.

Table II. *Ammonia production in the presence of glycine.*

Values in mg. NH_3-N

Anaerobic control	Anaerobic + glycine	Aerobic control	Aerobic + glycine
0·0275	0·0285	0·0800	0·200
0·0280	0·0302	0·1200	0·221
0·034	0·046	0·093	0·211
0·045	0·052	—	—
0·076	0·075	—	—
—	—	0·093	0·120
—	—	0·078	0·135

Under anaerobic conditions there was no increased ammonia production in the glycine-containing medium (Table II). The presence of oxygen was obviously necessary before the reaction could take place, and we were therefore inclined to assume that the increased ammonia formation in air represented an oxidative deamination of the glycine.

In order to obtain the ratio of the extra ammonia production from glycine to the extra oxygen required for deamination, measurements of oxygen uptake were made in the differential Barcroft apparatus. The ammonia was afterwards estimated in the contents of the Barcroft cups. We now found that the increased ammonia production was not necessarily accompanied by an increased oxygen uptake. Sometimes there was a slightly increased uptake, but this was in no way comparable with the extra amount of ammonia formed.

Atmospheric oxygen is therefore not directly used in the formation of the extra ammonia which appears when glycine is added, although the reaction does not proceed in its absence. It is possible that deamination of the glycine might occur as a secondary reaction, as the result, for instance, of the formation of hydrogen peroxide by the tissue, and without the uptake of further oxygen being necessary. Two experiments were made in which liver catalase was added to the preparations, but the ammonia formation was not inhibited thereby. The kidney tissue itself contains a great deal of catalase, so that it is possibly very difficult to alter the course of any reaction by the addition of more enzyme from outside sources. We cannot therefore draw any conclusions as regards the mechanism of the ammonia formation with glycine.

On the assumption that the increased production of ammonia was due to the deamination of the glycine, we have attempted to identify the deaminated products, but so far with complete lack of success. Comparison was made by Hagedorn and Jensen's [1923] method of the reducing values of the control preparations and of those containing glycine. The tissue was precipitated with tungstic acid, as this was found not to affect the estimations if neutralised before these were carried out. Ammonia was estimated in a portion of the same filtrate. It was found that ammonia formation was not accompanied by any increase in the reducing power of the filtrate. Control tests suggest that the method might fail to detect small amounts of acetaldehyde, and we have not yet made a more definite attempt to identify this possible product.

The possibility that a volatile acid, formic, acetic or glyoxylic acid, might be formed from the glycine was next considered. Larger amounts of tissue than usual were taken for these experiments, and were ground and precipitated with metaphosphoric acid after shaking at 37° for 3 hours in the appropriate media. Small portions of the filtrate were used for ammonia estimations.

To the main bulk of the filtrates excess of washed silver oxide suspended in water was added in order to remove the chlorides present. The solution was made strongly acid by the addition of H_2SO_4, and filtered into a small

Claisen flask. A few drops of caprylic alcohol were added to prevent frothing, and a stream of air was sucked through for 20 minutes to get rid of any excess CO_2 that might be present in any of the samples. Finally, distillation *in vacuo* at about 60° was carried out, but we could never find any extra volatile acids in the preparations containing glycine. By the method used it was possible to estimate 1 cc. of 0·01 *N* acetic acid, and, judging by the ammonia produced in the glycine media, about twice this amount of extra volatile acid might have been expected.

We could conclude from these experiments that volatile acids other than CO_2 were not formed.

The estimation of CO_2 was carried out in the Barcroft cups by having two small glass tubes hooked on to the sodium hydroxide pots. One of these contained a small piece of filter-paper saturated with 10 % KOH to absorb the CO_2 produced and the other was half-filled with saturated oxalic acid. The tissue was shaken in the apparatus in the usual way for about an hour, the oxygen uptake being measured, and then the two small tubes were upset by gentle tapping and shaking was continued for 5 minutes. All the CO_2 absorbed by the KOH or dissolved in solution was driven off and the amount could be measured. In seven out of eight cases there was more CO_2 produced in the presence of glycine than by the tissue alone, but there was no good agreement between the extra CO_2 and the ammonia production, assuming that glycine is completely oxidised. These facts, which will be further investigated, suggest that part of the glycine may be broken down completely.

Effect of glucose and cyanide on ammonia production by kidney tissue.

Warburg, Posener and Negelein [1924] working with brain and carcinoma tissue, and Meyerhof, Lohmann and Meyer [1925] working with diaphragm, found that the aerobic production of ammonia was depressed by the addition of glucose. György and Röthler [1927] considered that the anaerobic production of ammonia by kidney tissue was also depressed by glucose, but they relied upon the addition of a little chloroform to maintain anaerobic conditions in their preparations. We tried the effect of added glucose upon the aerobic and anaerobic ammonia-producing systems of kidney tissue in order to discover whether they were both equally affected, or whether they could be distinguished from each other by the action of glucose upon them. The glucose was dissolved in Ringer's solution, various concentrations being used at different times, and 1 cc. of the solution was added to the Barcroft cup or anaerobic tube.

We found that glucose had an inhibiting action only on the aerobic production of ammonia. In some cases, where the concentration of glucose was high enough (0·02 *M*), the ammonia production was stopped entirely, so that the ammonia value for the aerobic tissue in the presence of glucose was exactly the same as that of the anaerobic control (Table III). The ammonia

production was never cut down below the anaerobic level and when added to an anaerobic tube glucose had no effect upon the ammonia content.

It would appear, therefore, that the type of reaction concerned in the production of ammonia anaerobically is not the same as that by which it is produced under aerobic circumstances, since the latter is inhibited by glucose, and the former is not. This result agreed with that obtained by Loebel [1925] when working with the grey matter of brain.

Table III. *Effect of glucose on ammonia production.*

Values in mg. NH_3-N.

Glucose	Resting	Anaerobic + glucose	Anaerobic control	Aerobic + glucose	Aerobic control
M/50	0·01	—	0·033	0·033	—
M/50	0·0107	—	0·0279	0·0276	—
M/150	—	0·0477	0·0449	0·0565	0·077
M/75	—	0·043	0·045	0·048	0·075

Moreover, it was next found that glucose did not inhibit the extra ammonia formation with added glycine, so that the system responsible for this, although an aerobic one, is apparently not identical with that by which ammonia is produced from the tissue in the absence of glycine.

Watchorn and Holmes [1927] found that the formation of ammonia and urea by embryo kidney tissue growing *in vitro* could be completely inhibited by the presence of glucose.

Table IV. *Effect of glucose on ammonia production with glycine present.*

Values in mg. NH_3-N. All experiments aerobic.

	Without glucose			With glucose		
Exp.	Control	Glycine	Difference	Control	Glycine	Difference
1	0·089	0·205	0·116	0·053	0·190	0·137
2	0·077	0·189	0·112	0·052	0·148	0·096
3	0·093	0·211	0·118	0·060	0·190	0·130
		Av.	0·1153		Av.	0·121

Judging by the results obtained with anaerobiosis and by the addition of glucose, we are concerned with three types of ammonia-producing systems. One of these is an anaerobic system, whilst the other two are aerobic, and of the two aerobic systems, one is affected by glucose and the other is not. The distinction between these two latter systems might, of course, only be apparent and might be due to a difference in configuration of the substrate. We are ignorant of the nature of the substrate from which ammonia is formed in the absence of glycine. The results obtained when cyanide is added, do, however, once more suggest a distinction between the type of reaction by which ammonia is formed in the presence of glycine and that which causes the appearance of ammonia in the aerobic controls. The former is very much more sensitive to cyanide than the latter (Table V). The addition of enough cyanide (p_H 7·2) to bring the final concentration up to *M*/50 prevents any extra ammonia formation with glycine, and *M*/150 only allows a slight production.

Table V. *Effect of cyanide* (p_H 7·2) *on the formation of ammonia.*

NH_3-N in mg.

	Anaerobic control	Aerobic control	Aerobic +KCN	Aerobic + glycine +KCN	Aerobic +glycine
M/50 KCN	0·038	0·115	0·078	0·085	0·243
M/50 KCN	0·028	0·120	0·0708	0·0748	0·221
M/150 KCN	0·0275	0·080	0·0456	0·063	0·200

It can be seen from Table V that even *M*/50 cyanide, although diminishing the difference between the aerobic and anaerobic production of ammonia, does not prevent extra ammonia being formed in air by the tissue alone. On the other hand the ammonia production due to the presence of glycine is entirely inhibited, and the preparation containing glycine and cyanide gives the same ammonia values as that which contains no glycine (columns 4 and 5, Table V). The effect of cyanide on the anaerobic production of ammonia was tried only once, but as might be expected it did not alter this in any way.

It is not possible to say from these experiments which of these systems is responsible for the production of ammonia by the intact kidney for the neutralisation of acid glomerular filtrates. The greatest amount of ammonia is produced by the tissue in the presence of glycine but this may only be due to the high concentration of the substrate. It is hoped that a study of the effect of p_H upon the different systems will help to decide which of them is responsible for this function of the kidney.

Summary.

1. Washed kidney tissue produces some ammonia under anaerobic conditions, and more under aerobic conditions.

2. Glucose inhibits the aerobic formation of ammonia; the ammonia production is thus cut down to the anaerobic level in the presence of glucose. The anaerobic production is not affected.

3. In the presence of glycine extra ammonia is formed. This occurs only in air and not anaerobically, and yet no extra atmospheric oxygen is taken up by the tissue. The production of this extra ammonia is not inhibited by glucose.

4. Cyanide (0·02 *M*) diminishes but does not entirely inhibit the aerobic ammonia formation by the tissue alone. On the other hand, the production of extra ammonia which is due to the presence of glycine is entirely inhibited by 0·02 *M* cyanide.

5. Apparently three systems at least may be concerned in the ammonia production by kidney tissue, one anaerobic system and two aerobic. One of the aerobic systems is completely inhibited by glucose but not completely by 0·02 *M* cyanide, while the other is completely inhibited by cyanide and not at all by glucose.

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LXXXVII. THE GROWTH, DEVELOPMENT AND PHOSPHATASE ACTIVITY OF EMBRYONIC AVIAN FEMORA AND LIMB-BUDS CULTIVATED *IN VITRO*.

By HONOR BRIDGET FELL (*Senior Beit Memorial Research Fellow*) and ROBERT ROBISON.

From the Strangeways Research Laboratory, Cambridge, and the Biochemical Department, Lister Institute, London.

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Introduction.

It has been shown in previous work [Fell, 1928] that if terminal fragments of 8-day embryonic limb-cartilage with some adherent connective tissue were explanted *in vitro* they differentiated during subsequent cultivation into a diaphysial portion containing hypertrophied cells and an epiphysis containing smaller cells; in some cases bone was deposited around the diaphysial portion. If undifferentiated limb-bud mesenchyme were explanted [Strangeways and Fell, 1926; Fell, 1928] cartilage differentiated during cultivation, but with three exceptions [Fell, 1928] it failed to ossify and was of the small-celled type similar to that composing the normal avian epiphysis [Fell, 1925].

From these results it was clear that some at least of the chemical processes operating *in vivo* in the normal development of cartilage and bone also operated *in vitro* in explants of embryonic skeletal tissue. It seemed possible, therefore, that a study of the growth and differentiation of explants of embryonic cartilage and limb-bud mesenchyme could be correlated with a parallel study in the same material of certain chemical phenomena observed in normal and abnormal bones grown *in vivo*. It was thought that a combined morphological and biochemical study of such cultures might yield interesting information owing to the fact that experimental conditions can be more rigidly controlled *in vitro* than *in vivo* and tissue more easily handled and observed.

Bone and ossifying cartilage have been shown to contain a phosphatase [Robison, 1923], and this enzyme is regarded as an active agent in ossification, effecting the hydrolysis of certain phosphoric esters present in the blood [Kay and Robison, 1924; Martland and Robison, 1926] and thereby raising the concentration of inorganic phosphate in the tissue fluid so that calcium phosphate is deposited. In support of this hypothesis evidence was furnished

that the phosphatase is secreted in the region of the osteoblasts and hypertrophic cartilage cells. When severely rachitic bones were immersed in solutions of calcium hexosemonophosphate or calcium glycerophosphate, deposition of calcium phosphate took place in the periosteum and in the matrix of the proliferating and hypertrophic cartilage; on the other hand, no deposit was observed in the small-celled cartilage of the epiphysis [Robison and Soames, 1924, 1928]. Similarly, it was shown by examining the cartilages and bones of human embryos and young infants that, while the ossified portions of young normal bones contained phosphatase in high degree, the non-ossifying cartilage was in all cases devoid of the enzyme [Martland and Robison, 1924].

The present series of experiments was undertaken with two objects, (*a*) to study the correlation between the growth, development and phosphatase activity of isolated early embryonic femora and undifferentiated limb-buds cultivated *in vitro* and (*b*) to compare these processes in the explants with the corresponding processes in the normal embryonic limb.

Material and methods.

Material.

The material explanted consisted of the isolated femora of 5½ and 6-day fowl embryos and the entire limb-buds (anterior and posterior) of 3-day embryos.

Culture technique.

The tissue fragments were grown in a relatively large volume of medium contained in a watch-glass. The method[1] was as follows. A watch-glass, with its convex surface painted black to facilitate macroscopic observation, was placed on a layer of cotton wool at the bottom of a Petri dish 8 cm. wide and 1·5 cm. deep. After sterilisation 30–40 cc. of sterile distilled water was pipetted into the dish where it was absorbed by the cotton wool; in this way a highly effective moist chamber was produced. The culture medium, consisting of 10 drops of plasma and 10 drops of embryo extract, was then introduced into the watch-glass with capillary pipettes and allowed to clot. Either three or four explants were placed on the surface of the clot in each watch-glass, which was then incubated. The tissues were transferred to watch-glasses containing fresh medium every 3 days. This was effected in the following way. Each explant was first loosened from the clot either by lifting with a pair of fine forceps or by running the point of a cataract knife round the periphery of the cartilage so as to separate it from the zone of outgrowth; this latter method prevented the formation around the cartilage of a large mass of connective tissue and in the case of the femora minimised distortion during cultivation. The explants when loosened from the medium were sucked into a wide-bore pipette, washed in extract and with the same pipette placed on the surface of the clot in the new culture-vessel.

[1] This method is a modification of one tried by the late Mr. T. S. P. Strangeways.

The 3-day limb-buds after 3–4 days' growth usually became completely invested by ectoderm which subsequently keratinised. When the cultures were changed on the 6th day after explantation this layer of somewhat impervious keratin was dissected off with fine needles so that only a few small fragments remained attached to the connective tissue.

Chemical technique.

Phosphatase. Experiments were made to determine the most satisfactory method of estimating the phosphatase content of these small tissue fragments, having regard to the rather wide variations which might be anticipated both in their size and enzyme activity. For these control tests corresponding cartilages and bones from the right and left limbs of fowl embryos in varying stages of development were used. Extraction was carried out with chloroform water and with 0·1 % sodium bicarbonate solution for varying lengths of time, both the extracts and the extracted cartilage being separately tested for phosphatase by measuring the hydrolysis of sodium glycerophosphate under standard conditions. The results showed that extraction of the enzyme from these entire cartilages was very slow and incomplete, and that higher and more comparable values were obtained by allowing the cartilage itself to remain in the glycerophosphate solution during the test. The effect of varying the concentration of the substrate was also investigated. As the result of these experiments the following method was adopted for the estimation of phosphatase in the cartilages cultivated *in vitro*.

The tissue fragments were placed in small stoppered tubes containing 2 cc. distilled water saturated with chloroform, in which they were allowed to remain at ordinary temperature for 24 hours being meanwhile transferred from Cambridge to London. 1 cc. of 0·2 *M* sodium glycerophosphate (p_H 8·7) was then added to each tube and 1 cc. of the mixed solution immediately removed for the estimation of inorganic phosphate by the Briggs colorimetric method. The p_H of the remaining 2 cc. containing the tissue fragments was measured by means of the capillator, and, if necessary, was adjusted to 8·5–8·6. The tubes were then placed in a thermostat at 37° for 24 hours, after which the inorganic phosphate was again determined in 1 cc. of the solution, the increase representing the amount of hydrolysis effected by the tissue phosphatase. The 0-hour samples contained only traces of inorganic phosphate (0·001–0·002 mg. P), but some estimate of this was necessary in order to detect small amounts of hydrolysis or confirm its absence after 24 hours.

In calculating the phosphatase activity of the cartilage the assumption was made, based on control experiments, that only 20 % of the enzyme was present in the aqueous extract when the glycerophosphate was added, one-third of this 20 % being, therefore, removed in the 0-hour sample. Any error involved in this assumption will probably lie within the general limitations of the method. The increase of inorganic phosphate (mg. P) in 1 cc. × 2·14

gives, therefore, a measure of the hydrolysis effected by the tissue phosphatase in 24 hours at 37° and p_H 8·5. This value is denoted by *A*.

For the investigation of phosphatase production during growth of the embryonic bones *in vivo* a slight modification of the above method was considered advisable in view of the much larger size and phosphatase activity attained by the bones. Parallel estimations were carried out on femora from several embryos at each stage of development, the quantity of glycerophosphate being suitably increased for the larger bones. The 0-hour estimations were omitted, the necessary small corrections being found from control experiments with femora of similar age.

Weight. It was desired to correlate the phosphatase activity with the weight of the cartilage, but the determination of the moist weight was found to be impracticable owing to the very rapid evaporation from the surface of these minute fragments. It was also thought undesirable to dry the tissues before carrying out the phosphatase estimations, but a practicable compromise was found in the determination of the air-dry weight of the cartilage after the completion of the enzyme test. The tissues were removed from the glycerophosphate solution, rinsed with distilled water and the surface moisture removed with hardened filter paper. They were then placed on a coverslip, which had been previously weighed on a micro-balance, and were left exposed in the balance case until the weight became constant. Control tests carried out on the right and left limb-buds and cartilages of the embryos showed that considerable loss in weight occurred during the extraction, the loss being relatively greatest in the case of the smallest (and youngest) tissues. Thus, the air-dry weights of corresponding femora of a 6-day fowl embryo, the first untreated, the second after extraction and phosphatase test, were 0·061 mg. and 0·016 mg. respectively, while for the femora of a 21-day embryo the weights were 68·5 mg. and 60·0 mg. Some portion of this loss in weight is due to removal of the soluble constituents of the tissue fluids but autolysis of the tissue proteins may also account for some of the loss. No correction has been made for this loss in weight but the probable effect of such correction is indicated in discussing the results.

Histological technique.

Explants were removed from the watch-glasses at different periods of cultivation and were fixed in Zenker's fluid. The explanted femora required for a study of gross anatomy, after being fixed in Zenker's fluid and washed in water and in alcoholic iodine in the usual way, were stained in dilute haematoxylin, and were then dehydrated, cleared in xylene or clove oil and mounted whole in glass cells containing Canada balsam. Explants for histological investigation were embedded in paraffin wax and cut into serial sections; the slides were stained with haematoxylin and van Gieson's stain, safranine and picro-indigo-carmine or with Mallory's triple stain.

PART I. THE GROWTH AND DEVELOPMENT *IN VITRO* OF ISOLATED FEMORA OF 5½-DAY AND 6-DAY EMBRYOS.

Growth.

The rate of growth of the femora during cultivation *in vitro* was studied by determining the increase in length at intervals of 3 days, the measurements being made by the aid of a micrometer eye-piece without removing the cartilages from the culture medium. For this experiment 16 femora of 5½-day embryos and 18 femora of 6-day embryos were used, cultivation being continued during 27 days. The growth curves shown in Fig. 1 are plotted from the average lengths of the femora, those in which serious curvature developed

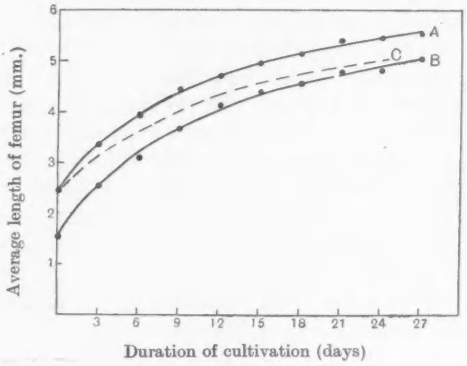

Fig. 1.

Curve A. Average increase in length of 6-day embryonic femora during 27 days' cultivation *in vitro*. Equation of curve: $y - 2{\cdot}47 = \frac{4{\cdot}5}{x+12}x$.

Curve B. Average increase in length of 5½-day embryonic femora during 27 days' cultivation *in vitro*. Equation of curve: $y - 1{\cdot}54 = \frac{5{\cdot}1}{x+12{\cdot}3}x$.

Curve C. Increase in length of 5½-day femora reckoned from the point at which their length was equal to that of the 6-day femora at 0-day. This length (2·47 mm.) was reached after 2·7 days' cultivation. Curve *C* is obtained from curve *B* by shifting each point 2·7 days to the left.

being omitted from the calculations. The 5½-day femora increased in length from 1·54 mm. to 5·03 mm. during 27 days while the 6-day femora increased from 2·47 mm. to 5·54 mm. during the same period. The absolute increase in length was, therefore, slightly greater for the 5½-day than for the 6-day femora. Relatively to their initial size, however, the 5½-day femora grew much more rapidly, increasing in length by 67 % during the first 3 days against 36 % for the 6-day femora, while in 27 days' cultivation the increase amounted to 226 % and 124 % respectively. The average weights (air-dry, after extraction) of the 5½-day and 6-day femora after 27 days' cultivation *in vitro* were 0·244 mg. and 0·482 mg. respectively.

The rate of growth *in vivo* was also determined by measuring the length of a number of femora of fowl embryos after periods of incubation from 6 to 21 days. In this series the length of the 6-day femora was 1·5 mm. and of the 9-day femora 6·0 mm., the increase during 3 days *in vivo* exceeding that during 27 days *in vitro*. At 21 days, the chicks having hatched, the average length of the femora was 23·3 mm. while the average dry weight was 60·0 mg.

Morphological development.

(*a*) *Anatomical.* The gross anatomy of the explants was studied partly by observation of the living cultures and partly by means of the series of whole mounts.

During cultivation *in vitro* the majority of the isolated femora maintained their characteristic shape to a remarkable extent (cf. Plate III, figs. 6 and 7).

When first explanted (0-day) the 5½-day femur of the normal embryo (Plate III, fig. 1) was a short thick rod showing at the distal end two rounded processes representing the developing condyles and at the proximal end two rather smaller knobs, one of which, projecting somewhat at right angles to the shaft, was the head and the other, more terminally placed, was the early trochanter.

After 3-days' cultivation (Plate III, fig. 2) the condyles had assumed a more definite shape whilst the head and trochanter were also more pronounced. The diameter at the middle of the shaft was only slightly larger than at the time of explantation but had considerably increased in the two epiphysial regions.

By about the 15th day of growth *in vitro* (Plate III, fig. 4) the characteristic pulley-like form of the condyles was very distinct. The head was also fairly well developed but the trochanter was usually less marked. The condyle, head and trochanter continued to enlarge during subsequent cultivation but showed no further changes in shape (Plate III, figs. 5 and 6).

During cultivation *in vitro* the explanted femora did not increase in width to the same extent in all regions (Plate III, figs. 2–6). After the 3rd day *in vitro* the diameter of the femur at the centre of the shaft increased very little and in 27-day explants was only about 25 % larger than at the time of explantation. On the other hand, the diameter of the proximal epiphysis in the same explants had usually enlarged by at least 130 % and the distal (condylar) epiphysis by at least 180 % of their original size. This difference between the relative growth rates of the epiphysial and diaphysial diameters was greater than in normal development, although *in vivo* also the percentage increase in diameter is much higher in the epiphysis than in the middle of the shaft. The relatively greater size of the epiphyses in the explanted femora was probably due partly to the limited periosteal ossification and absence of a marrow cavity. Ossification *in vivo* produces a mass of trabecular bone in the interstices of the network of periosteal blood vessels whilst the cartilage in the shaft is removed and replaced by the expanding marrow cavity.

In vitro, on the other hand, ossification gave rise only to a single compact layer of periosteal bone and the lateral expansion of the shaft could only have taken place by the growth of the highly differentiated, large-celled cartilage of which it was composed. It was also probable that the small-celled cartilage of the epiphysis actually grew more readily *in vitro* than the large-celled cartilage of the shaft; this was indicated by the fact that one of the commonest forms of abnormality encountered among the explanted femora was an attenuation of the shaft correlated with the presence of relatively large, mushroom-like epiphyses (Plate IV, fig. 12).

A minority of the explanted femora showed marked abnormalities, of which the commonest form has been described above. Twisting and distortion of the shaft sometimes occurred, which was due to two main causes. The first of these was the outgrowth of the epiphysial perichondrium into the fibrin clot; in some cases this outgrowth was so prolific as to anchor each epiphysis firmly to the medium so that increase in the length of the shaft could only take place by bending. The second cause of distortion was the formation around the shaft of extremely tough bands of fibrous tissue which, extending between and attached to the epiphyses, prevented the femur from elongating along its normal axis. As previously stated, this second factor could be largely eliminated by removing as much of the zone of outgrowth as possible when transferring the explants to fresh medium, and thus preventing the formation of an increasingly large mass of organised connective tissue around the femur.

The anatomical development of 6-day femora was also studied in the same way and gave very similar results to those obtained in the case of the $5\frac{1}{2}$-day specimens.

(*b*) *Histological.* The 6-day embryonic femora used for this part of the work were all slightly subnormal in development, having reached about the same stage as the $5\frac{1}{2}$-day specimens described in the preceding section.

In this series of cultures three femora were explanted in each watch-glass. Every three days one of the cultures was sacrificed, two of the explanted femora (the largest and smallest specimens) being used for the investigation of phosphatase activity and the third being fixed and sectioned. In this way it was possible to study the correlation between histological differentiation and enzyme activity; the latter aspect of the experiment is dealt with in the next section.

Sections of one of these 6-day embryonic femora (0-day) showed a very early type of cartilage (Plate IV, fig. 9). The shaft was better developed than the two ends and contained slightly larger cells separated by rather broader partitions of matrix. Most of the cells in the shaft appeared more or less oval with their long axes at right angles to the long axis of the femur but towards the end of the cartilage the chondroblasts were rounded and irregularly disposed. There was no sign of demarcation into epiphysis and diaphysis so that the young cartilage of the future epiphysis merged gradually into the

better developed cartilage of the future diaphysis. The periphery of the shaft was not very sharply defined but passed into a fairly broad, compact region of indifferent cells intervening between the cartilage on one side and the rudimentary muscle on the other. The outlines of the diaphysial regions were still less distinct and faded imperceptibly into a dense mass of undifferentiated mesenchyme. There was no trace of bone and no definite perichondrial membrane.

The femur fixed after 3-days' cultivation (Plate IV, fig. 10) showed a marked advance in development as compared with the control. The outline of the cartilage was much more distinct, the matrix was relatively abundant and the first indication of the three regions of cartilage cells—rounded, flattened and hypertrophic—characteristic of normal development [Fell, 1925] had appeared. The region of hypertrophied cells occupied about one-fifth of the total length of the cartilage; the cells were very irregular in form and the enlargement was not very great. Two zones of flattened cells compressed in the direction of the long axis of the shaft lay one on either side of the region of hypertrophy, each extending through about one-fifth of the length of the femur. The two ends of the cartilage contained smaller, rounded cells. There was no sharp border-line between the three zones which passed into each other very gradually. A perichondrium of somewhat elongated cells covered the surface of the femur and was continuous inwardly with the cartilage and outwardly with a thin layer of loose connective tissue.

After 6-days' cultivation the zone of hypertrophic chondroblasts had come to comprise about one-third of the length of the femur whilst the cells were much larger, rounded in form and showed the characteristic vacuolation of the cytoplasm. The flattened elements were much more plate-like than the 3-day explant and occupied a more sharply defined area which ended at the border of the epiphysial region containing small, rounded cells; there was thus a fairly distinct division into epiphysis and diaphysis. A very thin, conspicuous, rather sinuous sheet of early bone (possibly uncalcified) covered the surface of the cartilage in the region of the zone of hypertrophy. This sheet was overlaid by a layer of irregularly shaped cells resembling osteoblasts and these again were covered by a dense layer of spindle-shaped fibroblasts extending over the entire surface of the femur.

The specimen from the 9-day culture (Plate IV, fig. 11) showed no striking histological change.

In the 12-day explant the cartilage matrix was everywhere more abundant than in the younger specimens and the demarcation between epiphysis and diaphysis was more pronounced. The cartilage was enveloped by a layer of connective tissue which was thin and compact over the surface of the epiphyses and loosely reticular over the shaft. The zone of enlarged cells was overlaid by a thin layer of bone covered by a fairly well-defined periosteum.

The femur from the 15-day culture (Plate IV, fig. 12) was found to be

much less well developed than that fixed after 12 days *in vitro*; the reason for this imperfect development was not clear but it was probably due to some technical flaw in that particular culture. The region of hypertrophic cells was very restricted, there was no sharp division between epiphysis and diaphysis and an osseous layer could hardly be distinguished. As will be seen later, this subnormal differentiation was interesting in view of the fact that the two corresponding femora from the same culture showed a lower phosphatase activity than those from the 12-day culture.

In the femur fixed after 18 days *in vitro*, a comparatively stout layer of bone had been laid down on the surface of the cartilage around the zone of hypertrophy; this zone composed about one-half of the total length of the femur. The bone was thickest around the middle of the shaft where it contained typical branching bone-cells and was covered by a periosteum consisting of an outer fibrous and an inner osteoblastic layer. The two zones of flattened chondroblasts were relatively narrow and terminated abruptly at the borders of the small-celled epiphyses. A loose, highly fibrous mass of connective tissue surrounded the shaft and was continued over the articular surfaces of the epiphyses as a thin, compact perichondrium.

The femur from the 21-day culture (Plate IV, fig. 13) showed a much thicker layer of bone than was seen in the 18-day specimen. In one place the ossification had spread into the surrounding fibrous tissue and had formed a sheet of less densely staining (probably uncalcified) bony material running parallel with the sheath of periosteal bone covering the cartilage; a similar phenomenon was observed in an older (27-day) culture from the same series and in another 27-day culture from a different series. The cartilage itself showed little change.

Up to the 21st day *in vitro* the explants showed no more degeneration than is seen in the normal embryonic limb-skeleton; indeed, it was surprising to find that such a relatively large volume of tissue could remain so remarkably healthy under the conditions of life *in vitro*. In the 24-day explant, however, a limited number of shrunken cells were seen in places in both bone and cartilage, although elsewhere (Plate V, fig. 15) the tissue was still relatively healthy. This degeneration was still more extensive in the oldest (27-day) femur (Plate IV, fig. 14) which otherwise resembled the 21-day explant in histological structure; similar degenerative changes were seen in four 27-day cultures from another series. It seemed probable that this necrosis was due to an increasing density of the intercellular material of the cartilage, bone and connective tissue, which no doubt interfered with the proper diffusion of food material and excretory products and with the gaseous exchange.

In order to determine whether the osseous material laid down *in vitro* was calcified, four femora (from 5½-day embryos) from another series of cultures were fixed after 27 days' growth *in vitro* and were then stained whole by von Kossar's silver nitrate method. Of these one showed no blackening, another showed a single patch, whilst the remaining two displayed

a broad belt of calcification around the middle section of the shaft (Plate III, fig. 8).

In most of the older explants a few scattered muscle fibres of considerable length and showing beautiful cross striation were seen in the connective tissue surrounding the shaft. At the time of explantation only very simple early myoblasts were present so that the muscle fibres of the explants, which were often indistinguishable from isolated normal fibres, had differentiated during cultivation *in vitro*. This phenomenon will be investigated further.

For purposes of comparison a series of normal embryonic femora were histologically examined. It was found that differentiation proceeded more rapidly *in vivo* than *in vitro*, especially with regard to bone formation. In an 8-day normal femur, which would be equivalent in age to an explanted 6-day femur cultivated for 2 days, the region of hypertrophic cells was better developed than in the 3-day culture (equivalent in age to a 9-day normal embryonic femur) described above and was already covered by a two-layered periosteum and delicate sheath of bone similar to that seen in the 6-day culture. By the twelfth day of growth *in vivo* the periosteal bone had formed a fairly thick and highly vascular, trabecular layer enveloping the zone of hypertrophy whilst at the same time ingrowing blood vessels, connective tissue and marrow had begun to excavate the cartilage enclosed by the bone. In the equivalent 6-day culture, as previously mentioned, the bone formed merely a delicate membranous sheath around the middle segment of the shaft. On the other hand, the cartilage in the normal 12-day femur and in the equivalent 6-day culture was at almost the same stage of differentiation. The zone of hypertrophic cells occupied rather less relative space in the explant than in the normal bone, but the epiphyses were quite as clearly defined and the zone of flattened cells was on the whole rather more pronounced in the explant. By the eighteenth day of incubation two-thirds of the diaphysial cartilage of the normal femur had been excavated and replaced by marrow whilst the trabeculae of periosteal bone between the blood vessels were relatively long and stout. A marrow cavity was not formed in any of the femora explanted *in vitro* and ossification was never so advanced as *in vivo*; even in the oldest cultures the periosteal bone was represented by no more than the single compact layer described above in the case of the 21–27-day cultures.

Phosphatase.

The formation of phosphatase during growth *in vitro* of 6-day embryonic femora was investigated in parallel with the study of the histological development recorded above. Two femora from each culture were used for the phosphatase estimations but the values of A are given as the amounts of hydrolysis, in mg. P, effected by one femur in 24 hours under the specified conditions (p. 770). These values, plotted against the period of cultivation, are shown in Fig. 2, curve A.

The result of the 0-day test was completely negative, that is, the tissues before cultivation contained no phosphatase.

After 3 days' cultivation a definite though very small amount of phosphatase was found, $A = 0{\cdot}0026$; after 6 days', A had risen to 0·006; after 9 days' to 0·015 and after 27 days' to 0·135. An exception to the regular increase in phosphatase was shown by the 15-day culture, for which A was only 0·014 against 0·030 for the 12-day culture. It was noted in the previous section that the histological differentiation of the femur was much less advanced in the 15-day than in the 12-day culture.

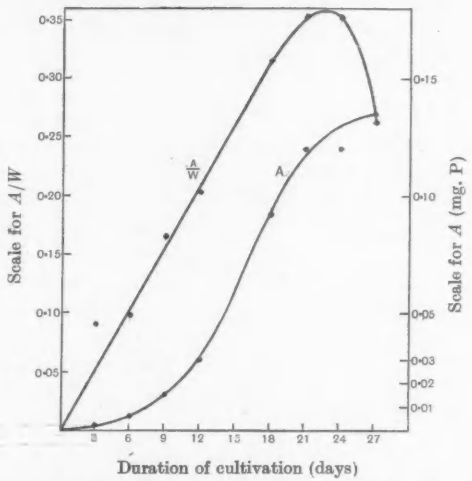

Fig. 2. Production of phosphatase during the development *in vitro* of femora from 6-day fowl embryos.

A = Phosphatase per femur; given as the amount of hydrolysis (mg. P) of sodium glycerophosphate in 24 hours at 37° and p_H 8·5.

A/W = Phosphatase per mg. dry weight of femur (weighed after extraction).

(The values of A and A/W for the 15-day culture, which was exceptionally backward in development, fall far below the curves and have been omitted for the sake of clearness.)

Weight. During 27 days' cultivation the dry weight of one femur (after extraction) increased from 0·014 mg. to 0·52 mg. Judging from the data of the control experiments, the corresponding dry weights of the unextracted femora would have been about 0·05 mg. and 0·9 mg. respectively. The femora of the 15-day culture were slightly heavier than those of the 12-day culture and in respect of weight, therefore, were not so backward as in histological development and phosphatase synthesis.

A/W. The ratio A/W expresses the amount of hydrolysis, as mg. P, effected in 24 hours by 1 mg. of tissue (dry weight). If phosphatase production is proportional to tissue growth, this ratio should be constant, but, if the enzyme is formed by hypertrophic cartilage and osteoblasts, and not by cartilage of the

early, small-celled type, the ratio should vary according to the degree of histological differentiation attained. The values of this ratio, plotted against the period of cultivation, are shown in Fig. 2, curve A/W. The value rises from zero at 0-day to a maximum of 0·35 after 21 days, remains constant for the next 3-day period and then falls to 0·26 at 27 days. Correction for the loss in weight of the femora during extraction would reduce all values of A/W, this reduction being relatively greatest for the youngest explants. The shape of the curve, however, would not be materially altered. (Compare the two curves A/W and A/W_1 for normal femora in Fig. 3.)

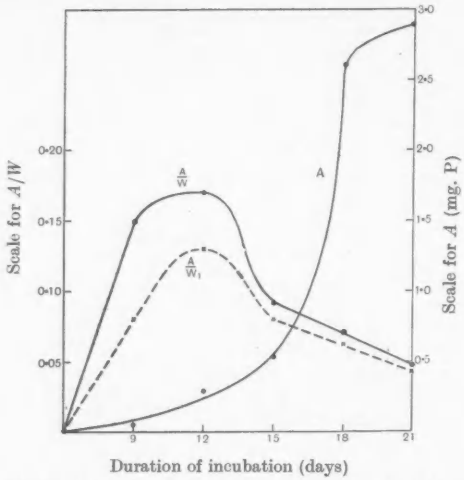

Fig. 3. Production of phosphatase in the femur of the embryonic fowl during normal development *in vivo*.

A = Phosphatase per femur.
A/W = Phosphatase per mg. dry weight of femur (weighed after extraction).
A/W_1 = Phosphatase per mg. dry weight of femur (calculated on dry weight of corresponding unextracted femur).

In order to compare these results with the rate of growth and phosphatase production *in vivo*, femora of normal fowl embryos were examined after the eggs had been incubated for different periods (3-day intervals) from 6 to 21 days. Phosphatase estimations were carried out in triplicate on single femora, one femur from each embryo being thus tested while the femur of the opposite limb was dried and weighed without extraction.

The increase in length (1·5 mm. to 23·3 mm.) of these normal femora between the sixth and twenty-first day *in vivo* has already been discussed (p. 772).

Weight. The average weight of one femur, after extraction, increased from 0·016 mg. at 6 days to 60 mg. at 21 days; the weights of the corresponding unextracted femora were 0·06 mg. and 68·5 mg. respectively.

Phosphatase. The values of A are plotted in Fig. 3, curve A. The 6-day femora were again devoid of phosphatase but by the ninth day *in vivo* the value of A had reached 0·045, increasing to 0·285 at the twelfth day and 2·90 at the twenty-first day (after hatching). By the twelfth day, therefore, the length, weight and phosphatase content of these normal embryonic femora considerably exceeded the maximum values attained by the explanted 6-day femora after 27 days' cultivation *in vitro*.

A/W. The ratio of phosphatase to the dry weight (A/W) rose from zero at the sixth day to a maximum of 0·17 at the twelfth day, thereafter falling to 0·05 at the twenty-first day. The values of this ratio are plotted in Fig. 3, curve A/W. The curve is of similar type to that showing the variation of this ratio during growth *in vitro* (Fig. 2), but differs from the latter in two respects, (*a*) the maximum value of A/W is reached in 12 days *in vivo* as against 6 days *in vivo* + 21 days *in vitro*, (*b*) this value is only 0·17 against 0·35 *in vitro*. The first point of difference is sufficiently explained by the more rapid rate of growth *in vivo*, the second can probably be accounted for by the presence in the normal embryonic femora of solid calcium salts and of marrow, which increase the weight without corresponding increase of phosphatase.

The curve A/W_1 (Fig. 3) also shows the ratios of phosphatase to dry weight, but here the weight, W_1, is that of the corresponding unextracted femur from the opposite limb of the same embryo. Assuming that the two femora were equal in weight this curve shows the effect of correcting the values of A/W, as usually obtained in these experiments, for the loss in weight during extraction and test. It is seen that the general shape of the curve is not greatly altered.

Part II. The growth and development *in vitro* of isolated limb-buds of 3-day embryos.

Growth and histological development.

The 3-day limb-buds (0-day) used in this experiment varied slightly in size in different embryos, the largest consisting of small rounded projections of the body wall whilst the smaller rudiments were somewhat flatter and more ridge-like. The leg-buds of each embryo were larger than the wing-buds. Histological sections showed no trace of cartilage or muscle and the buds were composed of undifferentiated mesenchyme.

After 21 days' cultivation *in vitro* the buds had enlarged considerably, giving rise to irregular nodules of cartilage adhering to the fibrin clot by an extensive outgrowth of perichondrial connective tissue. One explant from each of the five watch-glasses was fixed and sectioned whilst the remainder were tested for phosphatase activity.

The histological structure of the explants was almost precisely similar to that previously described by Strangeways and Fell [1926] in the case of the 3-day limb-buds cultivated in tubes. No bone or hypertrophic chondroblasts

occurred and the explants consisted of one or more nodules of small-celled cartilage, surrounded by a layer of fibrous connective tissue in which islets of keratinised ectoderm were sometimes seen (Plate V, fig. 16).

Phosphatase.

Eleven explants were tested for phosphatase after 21 days' cultivation and gave a completely negative result. The total dry weight of the cartilage after extraction was 0·36 mg. so that the amount of material was ample for the detection of the enzyme even if the relative activity of the tissue were only one-tenth of that of the 6-day femora cultivated for a similar period. Negative results were similarly obtained from four other cultures from 3-day limb-buds, the period of cultivation ranging from 9 to 41 days.

These experiments, therefore, confirm the view that phosphatase is not formed by cartilage of the small-celled type.

Discussion.

The self-differentiating capacity of the avian limb has already been demonstrated from the anatomical standpoint by Murray [1926] and Selby and Murray [1928]. These workers grafted limb-buds and fragments of limb-buds from embryos of 3–5 days on the chorio-allantoic membranes and found that the gross structure of the skeletal tissue which developed in the grafts was remarkably normal. Their results led them to conclude that the limb-rudiment of 3–5 day fowl embryos constitutes a self-differentiating, mosaic system. The experiments recorded in the present communication have shown that not only a fragment of the limb-bud but also a single cartilage segment, such as the femur, has a remarkable capacity for self-differentiation. When explanted *in vitro* by the technique described above, the early embryonic femur is completely deprived of a vascular system, nervous connections, adjacent skeletal structures and, if we except the few isolated muscle fibres scattered in the surrounding connective tissue, of association with the limb musculature. Nevertheless, as the results of the present investigation have shown, the explanted femur during cultivation *in vitro* is able to continue its anatomical development on the same general lines as in the normal limb, and at the same time undergoes a histological differentiation which is correlated with at least one of the chemical activities of the normal ossifying cartilage. The femora cultivated *in vitro* differed from normal embryonic femora of the same age mainly in their much smaller size, in having relatively larger epiphyses, in the absence of a marrow cavity and in being encased by a considerably thinner and more compact sheath of periosteal bone. All these differences may probably be attributed, in part at least, to the absence of certain mechanical and nutritional factors normally supplied by the blood vessels.

Throughout the whole period of growth *in vitro* the raw material required by the cells for the building up of new tissue must be obtained from the

culture medium by diffusion through the surface of the tissue fragment. But as the femur increases in size so the ratio of the surface area to the volume of tissue diminishes. To what extent the effect of this diminishing ratio on the food supply would account for the falling-off in the growth rate cannot be judged from our data. The curves shown in Fig. 1 give the increase in length only and these femora do not increase in the same proportion in all directions. Certain points, however, may be noted from these curves.

During cultivation the smaller $5\frac{1}{2}$-day femora increased in length more rapidly than the larger 6-day femora, both rates being measured from 0-day. If the increments are calculated in percentages of the 0-day lengths the difference is still more strikingly in favour of the $5\frac{1}{2}$-day femora. When, however, after 2·7 days *in vitro* the $5\frac{1}{2}$-day femora had reached the same length, 2·47 mm., as the 6-day femora at 0-day, their growth rate, calculated from this point (2·7 days), was actually slower than that of the 6-day femora calculated from 0-day. This is seen by comparing curves *A* and *C*. Curve *C* shows the growth rate of the $5\frac{1}{2}$-day femora reckoned from 2·7 days, that is, each point on curve *B* has been shifted 2·7 days to the left.

The regularity of these growth curves is interesting. Curves *A* and *B*, which fit the observed points very closely, are actually drawn from the equations of the hyperbolic curves:

$$A.\ y - 2{\cdot}47 = \frac{4{\cdot}5}{x+12}x. \qquad B.\ y - 1{\cdot}54 = \frac{5{\cdot}1}{x+12{\cdot}3}x.$$

For $x = \infty$ the values of y are 6·97 for curve *A* and 6·64 for curve *B*. These values of y correspond with the lengths of the femora at which growth ceases. For the working out of these equations we are indebted to Professor J. C. G. Ledingham, F.R.S.

The contrast between the histological development *in vitro* of the 6-day femora and the 3-day limb-buds is interesting. As in previous experiments [Strangeways and Fell, 1926; Fell, 1928] no differentiation into structures corresponding to the epiphysis and diaphysis took place in the cartilage formed *in vitro* from originally undifferentiated 3-day mesenchyme. On the other hand, the explanted 6-day femora which, at the time of explantation, were composed of very simple, early cartilage, developed epiphyses and diaphysis during the same period of cultivation. Thus, under the conditions of the experiments, the self-differentiating capacity of the 6-day femur was considerably greater than that of the 3-day limb-bud. The reasons for this fact are being investigated.

It has been shown that these femora, although entirely devoid of phosphatase when removed from the 6-day embryo, synthesise this enzyme during cultivation *in vitro* and that the production of the phosphatase follows very closely the progress of histological differentiation in the cartilage. Thus, the first appearance of phosphatase in the explanted 6-day embryonic femora, after 3 days' cultivation, coincided with the first appearance in the cartilage

of a zone of enlarged cells. The extension of this zone and increased enlargement of the cells in the later cultures were accompanied by a corresponding increase in the amount of phosphatase. The parallelism between enzyme synthesis and histological differentiation is, however, most clearly shown by considering the ratio of phosphatase to the dry weight of the femur (A/W). With the increasing proportion of hypertrophic cells and the gradual development of periosteum and periosteal bone observed up to the twenty-first day of cultivation there was found an increase in the value of this ratio, A/W, from zero to a maximum of 0·35. The degeneration which was revealed in the 24-day and still more extensively in the 27-day cultures was accompanied by a halt in the increase of this ratio followed by a fall to the value 0·26 at 27 days. Further evidence of this parallelism was provided by the chance inclusion in the series of one culture (15-day) in which, for some reason, the histological differentiation was very backward and for which the value A/W proved to be correspondingly low. On the other hand, it was shown that the cartilage formed during cultivation *in vitro* of 3-day embryonic limb-buds is entirely of the small-celled (undifferentiated) type and that even after 21 days' cultivation this cartilage synthesises no phosphatase.

The results of these experiments on the development *in vitro* of skeletal tissue confirm the view that phosphatase is synthesised by cartilage, only if hypertrophic cells are present.

The values for weight and phosphatase measured in this investigation and quoted above are, in some cases, of such small dimensions that it would be unjustifiable to claim for them any high degree of accuracy. For the older explanted femora and normal embryonic bones the amounts of hydrolysis were large enough for accurate estimation, but some caution is still needed in making the assumption that these amounts are proportional to the phosphatase present in the tissue. It is believed, however, that the possible errors, even on a generous scale, would not affect the significance of the results or invalidate the conclusions drawn from them.

The very considerable phosphatase activity attained by the explanted 6-day femora may be better appreciated if expressed in the following terms. The maximum value of A/W was 0·35, found after 21 days' cultivation *in vitro*. Under the conditions of the test, therefore, the amount of inorganic phosphate set free from the phosphoric ester by this femur in 24 hours was equivalent, in terms of calcium phosphate, $Ca_3(PO_4)_2$, to 1·75 times the dry weight of the femur. The corresponding values for the normal embryonic femora are 0·85 at 12 days and 0·24 at the time of hatching, or 0·65 and 0·22 if calculated on the dry weights of the unextracted femora.

SUMMARY.

1. The isolated femora of 5½-day and 6-day fowl embryos were found to have a remarkable capacity for self-differentiation.

2. During cultivation in a watch-glass the 5½-day femora increased to more than three times their original length.

3. During cultivation the gross anatomy of the femora remained comparatively normal.

4. The explanted femora underwent a remarkably normal histological differentiation. The three characteristic cell zones—small-celled epiphysis, intermediate belt of flattened cells and region of hypertrophic cells—appeared in the cartilage during cultivation. Periosteal bone and a periosteal membrane were also formed.

5. During cultivation *in vitro* the isolated femora synthesised a phosphatase; no phosphatase was present in the tissue at the time of explantation and the gross amount formed increased with the age of the culture.

6. The ratio of phosphatase to the dry weight of the femur increased during cultivation from zero to a maximum value and then declined. The increase corresponded with the progress of histological differentiation and the decline with degeneration. The maximum value of this ratio was higher than that found for normal embryonic femora.

7. Undifferentiated 3-day embryonic limb-buds cultivated *in vitro* gave rise to small-celled cartilage only.

8. Such explants after cultivation were completely devoid of phosphatase, thus confirming the view that the enzyme is formed by cartilage, only if hypertrophic cells are present.

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DESCRIPTION OF PLATES.

The outline of all the figures was drawn with the aid of either a camera lucida or a Leitz projection prism. All the figures on Plate III except Fig. 7 are drawn to the same scale and all the figures on Plate IV are to the same scale.

b., bone; *c.*, condyle; *ca.*, calcification; *c.t.*, connective tissue; *ep.*, epiphysis; *f.l.*, fibrous layer; *h.*, head; *h.c.*, hypertrophied chondroblast; *k.*, keratin; *my.*, myoblasts; *o.l.*, osteoblastic layer; *p.*, periosteum; *s.c.c.*, small-celled cartilage; *t.*, trochanter; *z.f.c.*, zone of flattened cells; *z.h.c.*, zone of hypertrophied cells.

PLATE III.

Fig. 1. Normal 5½-day embryonic femur as dissected for explantation (whole mount). Small processes representing the condyles, head and trochanter are present.

Fig. 2. Femur from 5½-day embryo after 3 days' cultivation *in vitro* (whole mount). The condyles, head and trochanter are more distinct.

Fig. 3. Femur from 5½-day embryo after 9 days' cultivation *in vitro* (whole mount).

Fig. 4. Femur from 5½-day embryo after 15 days' cultivation *in vitro* (whole mount).

Fig. 5. Femur from 5½-day embryo after 21 days' cultivation *in vitro* (whole mount).

Fig. 6. Femur from 5½-day embryo after 27 days' cultivation *in vitro* (whole mount). Note the relatively normal appearance of this femur as compared with the normal specimen (Fig. 7). It will be seen that the increase in width is much greater in the region of the epiphyses than in the middle of the shaft; this is more marked than in the normal femur.

Fig. 7. Normal femur from 21-day embryo (whole mount).

Fig. 8. Femur from 5½-day embryo after 27 days' cultivation *in vitro* (whole mount). This specimen has been stained by von Kossar's silver nitrate method. Note the sheath of calcified bone enclosing the middle region of the shaft.

PLATE IV.

Fig. 9. Section of normal femur from 6-day embryo. The femur is composed of a very simple type of cartilage which shows no hypertrophied cells and no differentiation into epiphysis and diaphysis. (Safranine and picro-indigo-carmine.)

Fig. 10. Section of a 6-day embryonic femur after 3 days' cultivation *in vitro*. More matrix is present than in the previous specimen and indications of a differentiation into epiphysis, zone of flattened cells and zone of hypertrophied cells are seen. (Safranine and picro-indigo-carmine.)

Fig. 11. Section of a 6-day embryonic femur after 9 days' cultivation *in vitro*. The region of hypertrophied cells is now fairly distinct and is overlaid by a delicate layer of very early bony material. (Haematoxylin and van Gieson's stain.)

Fig. 12. Section of a 6-day embryonic femur after 15 days' cultivation *in vitro*. This explant is subnormal in development as compared with other explants; the zone of hypertrophied cells is not quite so extensive as in the 9-day culture and there is no definite boundary between epiphysis and diaphysis. Note the large size of the epiphysial regions relative to the shaft. (Mallory's triple stain.)

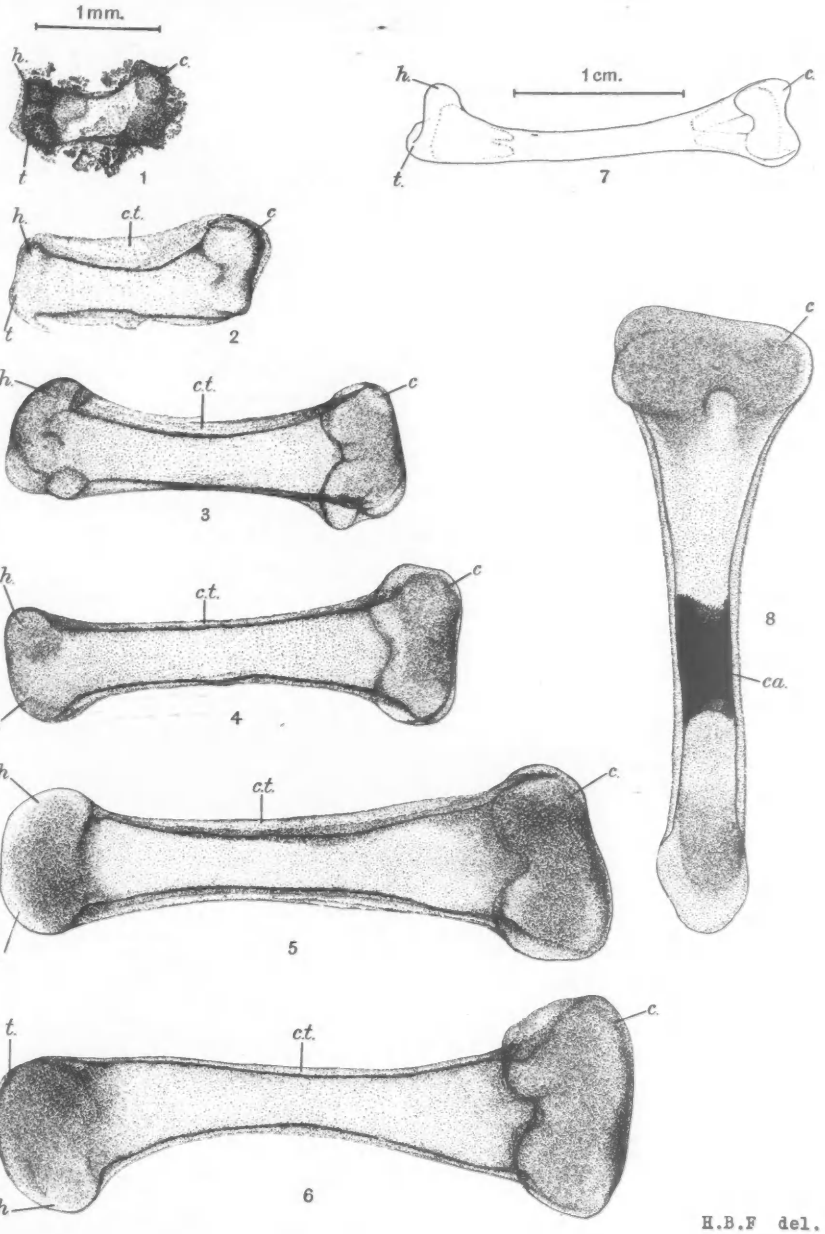
Fig. 13. Section of a 6-day embryonic femur after 21 days' cultivation *in vitro*. The epiphysis is sharply marked off from the diaphysis by the zone of flattened cells; a sheath of bone invests the extensive zone of hypertrophied chondroblasts. (Safranine and picro-indigo-carmine.)

Fig. 14. Section of a 6-day embryonic femur after 27 days' cultivation *in vitro*. (Safranine and picro-indigo-carmine.)

PLATE V.

Fig. 15. Section of a 6-day embryonic femur after 24 days' cultivation *in vitro*. Hypertrophied chondroblasts, bone and the two-layered periosteum are seen. (Safranine and picro-indigo-carmine.)

Fig. 16. Section of a 3-day embryonic limb-bud after 21 days' cultivation *in vitro*. An irregular mass of small-celled cartilage has been formed during cultivation; no bone is present. (Mallory's triple stain.)



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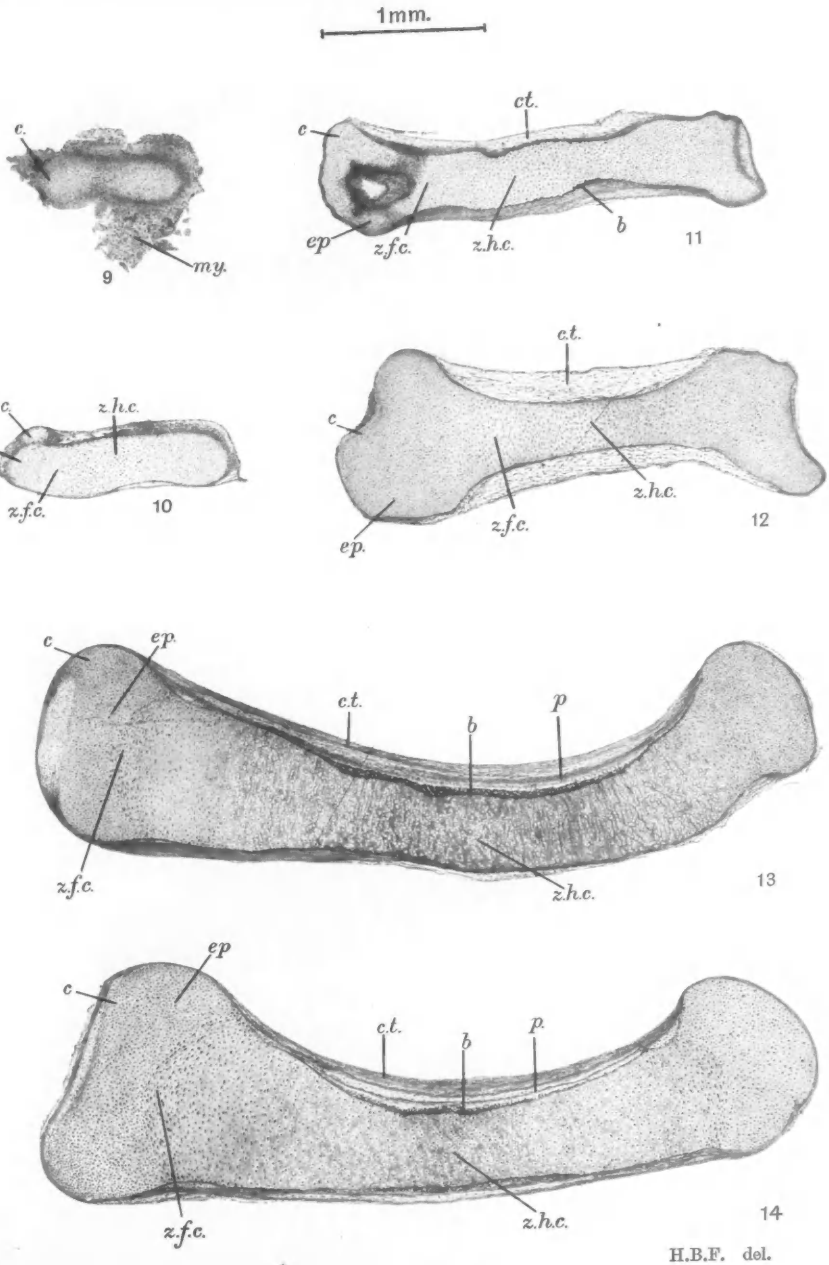
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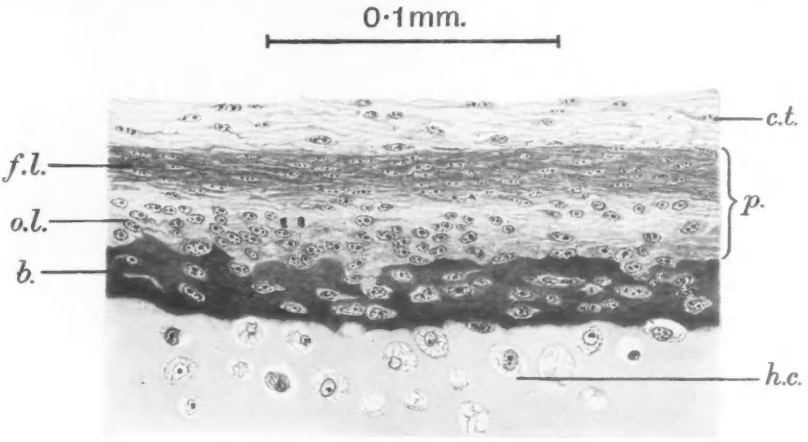

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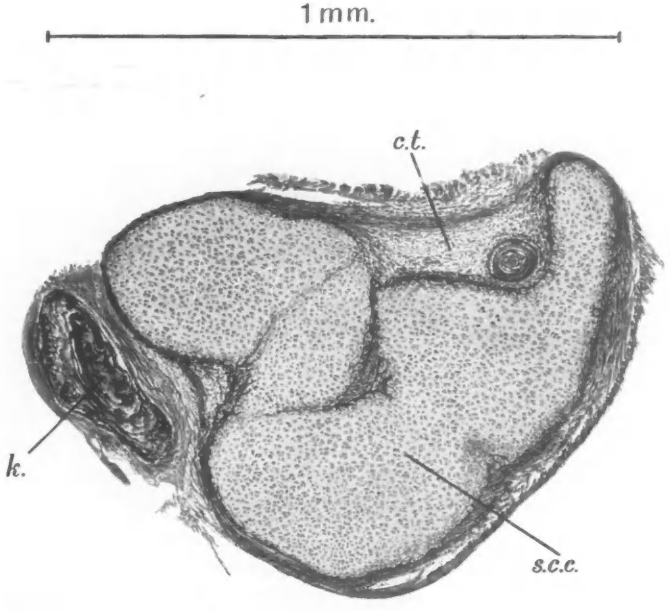

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LXXXVIII. OBSERVATIONS ON THE ASSAY OF VITAMIN A.

By JACK CECIL DRUMMOND and RICHARD ALAN MORTON.

From the Department of Physiology and Biochemistry, University College, London, and the Department of Chemistry, University of Liverpool.

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Introduction.

The assay of vitamin A presents a number of difficulties that have not yet been satisfactorily overcome. On the one hand, the biological method is subject to error on the ground of the wide variations that may be encountered, and against which few take adequate precautions. On the other hand, it is still uncertain whether the colour reaction of Rosenheim and Drummond, on which the alternative method of estimation is based, is due specifically to the vitamin.

The biological method has been reviewed several times recently, and on each occasion emphasis has been laid on the very disturbing variations in response exhibited by the animals. Although this is now somewhat generally recognised, it is, unfortunately, exceptional to find that an investigator has employed a sufficiently large group of animals to minimise the error arising from this cause.

The biological method has in the past usually been carried out by observing the effect of a measured supplement of the foodstuff on young rats whose development has been retarded by a period of feeding on a diet devoid of, or seriously deficient in, the vitamin. It seems highly probable that Hume and Smith [1928] are correct in attributing in such tests some of the variations in response to the ill-health that may arise during the period of depletion. Nevertheless, they still find rather disturbing variations when the test is made by supplying the substance under investigation from the beginning.

The necessity of using large groups of animals in either form of the biological test renders the undertaking of numerous comparative studies a formidable task. Such a position arose when one of the authors (J.C.D.) undertook, in conjunction with Prof. T. P. Hilditch, an extended investigation into certain aspects of the vitamin content of cod-liver oils on behalf of the Empire Marketing Board. In the course of this study it became necessary to examine several hundred samples for vitamin A, and to have submitted them all to an adequate biological test would have proved a very costly and laborious matter. The alternative was to rely on other and less definitely specific tests if it could satisfactorily be proved that they give results in agreement with the animal experiments. The method of estimation based on

the colour reaction of Rosenheim and Drummond has, during several years, proved in our laboratory very reliable, particularly in the examination of cod-liver oils.

A number of statements have, however, appeared in the literature to the effect that the colorimetric method of assay is untrustworthy. It will be shown in a later section of this paper that these statements are generally founded on unsatisfactory evidence.

The Accessory Food Factors Committee of the Medical Research Council arranged a comparative test between the biological method of assay, as used in a number of different laboratories, and the colorimetric method. The report of this test [1928] stated that the latter method afforded evidence consistent with that derived from the animal experiments, but its greatest value was to demonstrate clearly for the first time the serious nature of the variations encountered in the biological method as usually employed. The Committee was forced to admit that the biological methods employed were not sufficiently accurate to permit the results being presented in numerical form.

In view of the task that faced us, it seemed desirable that another and much more severe comparative examination of the two methods should be made before finally accepting or rejecting the colorimetric method as a routine test. Furthermore, it seemed desirable that the comparison should be extended to cover a method based on the quantitative examination of the absorption band in the ultra-violet spectrum extending from 280 to 360 $\mu\mu$ (max. near 328 $\mu\mu$) believed by Morton and Heilbron [1928] to be characteristic of vitamin A.

For the purpose of making this extensive test six samples of cod-liver oil were selected at random from our stock of recently collected materials. The main part of this report records the investigations on these six oils, but a number of other materials containing vitamin A were also examined by the three methods, although in these cases the biological tests were not made as thoroughly. The tests were conducted in the following manner.

(*a*) Biological tests.

The "curative" or "recovery" test was employed throughout. The investigation was well advanced before Hume and Smith [1928] pointed out the disadvantages of this method, and advised dispensing with the depletion period.

The rats employed in these tests were bred in the laboratory from stock fed on a mixed diet that has supported satisfactory growth and reproduction for many generations. At an average weight of 45 g. they were placed on the vitamin A-free diet compounded as follows:

Purified caseinogen	...	...	20
Purified rice starch	...	...	72
Salt mixture	...	...	4
Yeast extract	...	...	4

Vitamin D was supplied in the form of irradiated ergosterol prepared in the laboratory. A dose of 0·01 mg. of this preparation dissolved in ethyl oleate was given twice weekly to each rat.

We have found ethyl oleate a convenient solvent for materials that must be diluted before administration. The choice of a suitable diluent has not proved an easy matter. It is, in our opinion, inexcusable to use natural oils, such as arachis oil, for such a purpose. To do so is to run a very grave risk of disturbances caused by the action of components having unsuspected physiological activity.

On the other hand, medicinal paraffin, used by Drummond, Coward and Handy [1925], is not entirely satisfactory, in view of the uncertainty that absorption of the dissolved substance is quantitative.

Ethyl oleate has been found more satisfactory in this respect, although not wholly ideal. Many of the natural oils contain substances that appear to act as "anti-oxydants," capable to some extent of protecting labile substances such as vitamin A. The nature of these substances is unknown, but they are removed during our preparation of ethyl oleate, so that, unless care is taken to keep the solutions out of contact with oxygen, a more rapid destruction of vitamin A tends to occur in this solvent than in a natural oil.

The preparation of the ethyl oleate from a high-grade olive oil is a simple matter, the most important step being the complete removal of the unsaponifiable constituents by ether extraction of the soaps before separating the fatty acids. In view of the trouble caused in the past, this step is essential in case there should be present substances possessing physiological action. The ethyl esters prepared from the purified fatty acids are fractionated under reduced pressure, only the main fraction being used. It is an excellent solvent, and is readily taken by the rats. It is possible that the rate of loss of vitamin A dissolved in this solvent and exposed to air might be greatly retarded by the addition of a suitable "anti-oxydant" such as quinol. This is being investigated. Meanwhile we use brown glass ampoules of small capacity and store them while the contents are being used for feeding in a vacuum desiccator in a cold, dark cupboard. Unopened the contents keep well.

Each rat was kept in a separate cage and the supplements of vitamin A were in every case administered directly into the mouth. In the case of the experiments on cod-liver oil, groups of not less than seven rats were employed for each dosage and at least four different doses of each oil were given. Smaller groups of about four animals were used for most of the doses of the other substances investigated.

The problem of presenting the results of these biological tests is a difficult one, which raises afresh the much-discussed point as to the most satisfactory manner in which to express the response of the animals. Several workers of experience have criticised one method often used, namely, comparison of the rates of growth evoked by the same dose of different oils. It is obviously less misleading to compare the doses necessary to induce the same rate of growth,

provided that this is subnormal. In any case the test must not be too prolonged, for even on doses producing normal or nearly normal growth in the early stages for a time, the response may later show a failure which is not made good by subsequently increasing the amount of the supplement.

The opinion appears to be general that it is reasonably safe to compare the growth for a period of 5 to 6 weeks after giving the supplement.

In the experiments described in this paper it has proved difficult to decide at what stage to make the comparison. The majority of the experiments extended over 5 or 6 weeks, but in a few cases results based on shorter or longer periods have been included in the tables. Another difficulty concerns deaths during the course of the tests. In the results given below, records of animals that died before they had received the supplements for 3 weeks have been excluded. Such decisions are doubtless open to criticism, but it is not easy to decide on a more satisfactory plan.

The results on the six cod-liver oils are presented below.

Table I. *Oil C.*

Dose (mg.)	No. of rats	Reference No.	Period on test (weeks)	Change in wt. (g.)	Change in wt. per week (g.)	Average for group (g.)
10	6	L 20/74	6	+42	+ 7·0	+6·1
		L 20/77	6	+40	+ 6·6	
		21/71	5	+10	+ 2·0	
		L 99/75	5	+51	+10·2	
		L 99/76	5	+36	+ 7·2	
		L 99/74	6	+22	+ 3·7	
7	8	L 99/70	5	+12	+ 2·4	+4·4
		L 99/71	5	+27	+ 5·4	
		L 97/78	5	+27	+ 5·4	
		L 97/79	5	+16	+ 3·2	
		L 97/77	5·5	+32	+ 5·8	
		L 97/76	5	+30	+ 6·0	
		L 96/62	5	+22	+ 4·4	
		L 96/63	6	+16	+ 2·7	
5	6	L 96/61	5	+36	+ 7·2	+2·8
		L 96/60	6	−10	− 1·7	
		L 96/59	6	− 8	− 1·3	
		L 98/82	6	+22	+ 3·7	
		L 98/81	5	+12	+ 2·4	
		L 98/80	5	+16	+ 3·2	
2	8	L 100/92	5	+20	+ 4·0	+1·5
		L 100/93	6	+20	+ 3·3	
		L 100/94	6	+22	+ 3·7	
		L 100/95	6	−10	− 1·7	
		L 98/83	5	0	0	
		L 98/84	5·5	+ 5	+ 0·9	
		L 101/100	5·5	+ 2	+ 0·4	
		L 101/101	5	+ 6	+ 1·2	

The analysis of these results is not simple. If the principle of comparing the doses necessary to promote the same rate of growth (subnormal) be followed, it is convenient to adopt the rate of 3 g. per week suggested by Sherman and used in the official method of assay published in the United States Pharmacopoeia (X) 1926.

Table II. *Oil F.*

Dose (mg.)	No. of rats	Reference No.	Period on test (weeks)	Change in wt. (g.)	Change in wt. per week (g.)	Average for group (g.)
10	7	210	6	+48	+ 8·0	+8·5
		213	6	+45	+ 7·5	
		214	6	+44	+ 7·3	
		L 40/180	5	+58	+11·6	
		L 39/174	5	+35	+ 7·0	
		L 39/175	5	+49	+ 9·8	
7	8	L 31/132	5·5	+15	+ 2·7	+5·5
		L 37/167	5·5	+16	+ 2·9	
		209	7	+34	+ 4·9	
		212	5·5	+40	+ 7·3	
		L 39/172	5·5	+15	+ 2·7	
		L 39/173	5	+20	+ 4·0	
		215	5	+60	+12·0	
		L 38/168	5	+32	+ 7·4	
5	9	218	4·5	+27	+ 6·0	+4·3
		L 34/135	5	+23	+ 4·6	
		L 35/152	6	+14	+ 2·3	
		L 20/78	5·5	+25	+ 4·5	
		L 21/83	6	+21	+ 3·5	
		208	5·5	+17	+ 3·1	
		L 34/144	5	+20	+ 4·0	
		207	5·5	+32	+ 5·8	
		L 29/123	6	+31	+ 5·2	
2	6	L 20/75	6	+19	+ 3·2	+2·3
		L 21/91	5	+14	+ 2·8	
		231	5	+ 9	+ 1·8	
		232	5	+28	+ 5·6	
		L 20/74	3	−10	− 3·3	
		L 21/82	5·5	+19	+ 3·5	

Table III. *Oil G.*

Dose (mg.)	No. of rats	Reference No.	Period on test (weeks)	Change in wt. (g.)	Change in wt. per week (g.)	Average for group (g.)
10	6	L 47/210	5·5	+29	+ 5·3	+5·8
		L 48/219	5	+34	+ 6·8	
		L 46/203	5	+19	+ 3·8	
		L 46/207	7·5	+40	+ 5·3	
		L 49/223	7·5	+38	+ 5·1	
		298	5	+43	+ 8·6	
7	6	L 41/185	5	+57	+11·4	+4·7
		L 43/195	4	−24	− 6·0	
		L 46/206	5	+21	+ 4·2	
		297	5	+35	+ 7·0	
		L 43/197	5	+34	+ 6·8	
		299	5	+25	+ 5·0	
5	4	L 43/192	5·5	+18	+ 3·3	+3·7
		L 49/221	5	+38	+ 7·6	
		L 49/222	6	+22	+ 3·7	
		301	5·5	+ 1	+ 0·2	
2	12	L 40/182	6	+17	+ 2·8	+1·6
		L 40/178	5	+10	+ 2·0	
		L 39/176	5	+29	+ 5·8	
		L 39/177	5	− 2	− 0·4	
		L 39/170	5·5	+19	+ 3·4	
		L 38/171	5	−10	− 2·0	
		L 7/17	5	+ 3	+ 0·6	
		L 7/16	5	− 4	− 0·8	
		L 34/146	5	+22	+ 4·4	
		L 34/147	5·5	+ 7	+ 1·3	
		L 37/166	4	−11	− 2·8	
		L 37/165	5	+25	+ 5·0	

Table IV. *Oil J.*

Dose (mg.)	No. of rats	Reference No.	Period on test (weeks)	Change in wt. (g.)	Change in wt. per week (g.)	Average for group (g.)
10	7	L 36/158	5	0	0	+2·6
		L 36/156	5	+13	+ 2·6	
		L 35/152	6	+14	+ 2·3	
		224	6	+26	+ 4·3	
		226	5	+25	+ 5·0	
		227	5	+20	+ 4·0	
		L 36/157	5	− 6	− 1·2	
7	8	L 36/161	6	+ 3	+ 0·5	+4·0
		L 37/162	5	+26	+ 5·2	
		L 36/160	5	+ 7	+ 1·4	
		L 36/159	5	+ 7	+ 1·4	
		220	6	+27	+ 4·5	
		223	5	+ 3	+ 0·6	
		221	6	+37	+ 6·1	
		222	5	+63	+12·6	
5	8	L 30/125	6	+11	+ 1·9	+2·8
		L 30/127	6	+43	+ 7·1	
		L 32/134	6	+34	+ 5·7	
		L 32/136	6	+42	+ 7·0	
		L 37/163	5	− 7	− 1·4	
		L 37/164	5	−12	− 2·4	
		217	4	+12	+ 3·0	
		218	3	+ 6	+ 2·0	

Table V. *Oil K.*

Dose (mg.)	No. of rats	Reference No.	Period on test (weeks)	Change in wt. (g.)	Change in wt. per week (g.)	Average for group (g.)
10	6	L 41/187	5	+39	+ 7·8	+5·8
		L 42/193	6	+39	+ 6·5	
		L 47/211	5	+50	+10·0	
		L 48/216	5	+24	+ 4·8	
		L 47/213	5·5	+33	+ 6·0	
		L 42/198	5	0	0	
7	6	L 43/196	5	+12	+ 2·4	+3·2
		L 46/204	7	+46	+ 6·6	
		L 48/215	6	+28	+ 4·6	
		L 49/224	6	+29	+ 4·8	
		L 76/284	6	− 4	− 0·7	
		L 76/299	5	+ 7	+ 1·4	
5	6	L 41/186	5	+42	+ 8·4	+2·5
		L 42/192	4	− 4	− 1·0	
		L 50/227	5	+15	+ 3·0	
		L 50/228	5·5	+ 5	+ 1·0	
		L 55/237	5	− 6	− 1·2	
		L 72/278	5	+23	+ 4·6	
2	6	L 55/238	5	− 8	− 1·6	−0·6
		L 55/239	5	− 6	− 1·2	
		L 72/277	5	+22	+ 4·4	
		L 63/268	4	−13	− 3·2	
		L 72/279	5	− 4	− 0·8	
		L 67/282	5	− 5	− 1·0	

The individual variations are surprising, particularly when it is observed that many of the animals showing wide differences in behaviour on the same dosage are litter mates. Numerous examples will be seen (*e.g.* ♂ 195 (Litter 43) given 7 mg. of oil G loses 24 g. in 4 weeks, whilst a sister (♀ 197) grows 34 g. in 5 weeks).

Table VI. *Oil L.*

Dose (mg.)	No. of rats	Reference No.	Period on test (weeks)	Change in wt. (g.)	Change in wt. per week (g.)	Average for group (g.)
10	6	L 41/189	5	+20	+4·0	+2·0
		L 47/212	5	− 1	−0·2	
		L 33/139	6	+31	+5·0	
		L 22/122	6	+12	+2·0	
		L 30/126	5·5	+ 7	+1·2	
		L 30/128	5·5	0	0	
7	3	L 41/188	5	+20	+4·0	+2·9
		L 46/208	5	+13	+2·6	
		L 49/225	3	+ 7	+2·3	
5	4	L 48/217	5·5	+ 9	+1·6	+0·9
		L 48/213	4·5	+ 4	+1·0	
		L 33/140	5	− 7	−1·7	
		L 33/138	5	+13	+2·6	
2	6	L 31/130	5	− 5	−1·0	+0·4
		L 32/133	5	− 3	−0·6	
		L 32/135	6	+ 1	+0·2	
		L 32/137	5·5	+ 5	+1·0	
		L 35/150	5	+12	+2·4	
		L 35/151	5	+ 2	+0·4	

But even when the results for each group are averaged, a more serious type of discrepancy remains. This is shown clearly in the lower average rate of growth on 10 mg. of the oil J than on the dose of 7 mg. The same difficulty arises in the case of oil L. Which of the values for these oils should be accepted? The choice presents an opportunity for doubling the possible vitamin value of the oil in the former case. If, however, as seems most reasonable, we accept the still lower dose (5 mg.) of the oil J, the following doses are found to promote a rate of growth of approximately 3 g. a week for a period of 5 to 6 weeks:

	mg.	Relative vitamin value
C	5	2
F	2	5
G	<5	>2
J	5	2
K	7	1·5
L	10*	1

* The value of 10 is accepted in this case because of the small number of animals in the group to which the dose of 7 mg. was given. Four deaths occurred in this group before 3 weeks had passed. The oil was undoubtedly the poorest in vitamin A of the six examined.

A survey of the figures in Tables I–VI does not encourage complacency with regard to the value of the biological test. Obviously even larger groups than 7 to 12 animals and a more extensive range of doses would have to be employed if the accuracy of the method were to be increased so as to distinguish with certainty between two oils showing less than a 100 % difference in vitamin A content.

If the figures we have presented are examined on the alternative basis, namely, a comparison of the rate of growth produced by the same doses of the different oils the following results are obtained[1].

[1] The values for the 2 mg. doses have been excluded as they are obviously unsuitable for treatment on these lines.

Table VII. *Comparison of six oils (biological tests).*

Normal rate of growth at period covered by tests (80–130 g.): ♂ 14·5 g./wk.; ♀ 10·3 g./wk.

	Average weekly weight gain (g.) on dose			Relative values L = 1 on dose		
Oil	10 mg.	7 mg.	5 mg.	10 mg.	7 mg.	5 mg.
C	6·1	4·4	2·8	3·0	1·5	3·1
F	8·5	5·5	4·3	4·2	1·9	4·8
G	5·8	4·7	3·7	2·9	1·6	4·1
J	2·6	4·0	2·8	1·3	1·4	3·1
K	5·8	3·2	2·5	2·9	1·1	2·8
L	2·0	2·9	0·9	1	1	1

These figures are entirely unsatisfactory from a quantitative standpoint, but they could be interpreted as indicating that L is the poorest oil, G and F the best with 2 to 4 times the activity of L, whereas the other three, K, J and C are intermediate in value. As a matter of fact, if the average of the relative values for each oil on the three doses of 10, 7 and 5 mg. are taken, a rather questionable procedure, the figures are more or less of the same order as those yielded by the comparison of the doses required to produce a weekly gain of 3 g.

Oil	Relative value deduced from dose necessary to promote growth at approximate rate of 3 g. a week	Relative value deduced from average of relative values on 10, 7 and 5 mg. doses in Table VII
C	2	2·5
F	5	3·6
G	>2	2·9
J	2	1·9
K	1·5	2·3
L	1	1

The biological assay of these six oils has been carried out with more care than is usually bestowed on such tests. No less than 190 animals were employed, and yet the results do not enable us to judge the relative values of the oils more than approximately. F would certainly appear to be the oil richest in vitamin A, probably about 4 or 5 times more potent in this respect than the poorest oil L. Of the other four it is only possible to state that they possess intermediate values, and that G is probably second richest.

Our own conclusion from this study of the biological test is that by using reasonably large groups of rats (7–12) drawn from a uniform stock, it is possible to detect differences of the order of 100 % in the vitamin A content of cod-liver oils. This refers of course only to the "curative" or "recovery" form of the test. It is possible that the alternative method advised by Hume and Smith would be more sensitive.

(*b*) Colorimetric tests.

The six oils were examined by the Carr and Price modification [1926] of the Rosenheim and Drummond test. At least three independent determinations by the Lovibond tintometer were made on each oil. Determina-

tions of the blue colours were also made by means of a Hilger-Nutting spectrophotometer, attached to a constant deviation wave-length spectroscope. This is probably the most accurate method available for the absolute measurement of the intensity of absorption at the head of the band responsible largely for the blue colour.

The blue colour to be measured suffers from the disadvantage of being transient, and special precautions had to be taken to ensure the recording of the maximum intensity of absorption. The type of cell used is specially well adapted for spectroscopic work with transient colours. It is made in one piece of fused quartz with flat fused-on end plates. Two such cells are placed empty in the cell holders and the zero adjustment of the photometer checked. The comparison cell, without being removed from the cell holder, is then filled with the solution of antimony trichloride. Using a specially calibrated pipette with a fine jet, 0·3 cc. of a solution of the cod-liver oil, at an appropriate dilution in chloroform, is run into the other cell. The observer then adjusts the drum of the wave-length spectrometer to 608 $\mu\mu$, the head of the sharp absorption band, in preparation for making rapid measurements. A second worker then allows 3 cc. of the 30 % solution of antimony trichloride to run into the absorption cell, tapping it to secure a homogeneous mixture. The observer matches the intensity of the blue colour every 15 seconds, the readings being recorded by his colleague.

The photometer readings are in terms of $\log I_0/I$; I_0 being the intensity of the light incident on the absorption cell and I that of the emergent light. When $\log I_0/I$ is plotted against time, the absorption at 608 $\mu\mu$ reaches a maximum after about one minute. If the maxima in these curves are compared for different samples (equating the concentrations and cell thicknesses), the relative values express the relative intensities of the blue colours for the different samples. This procedure, with practice, gives trustworthy results.

The relative values obtained by the two modifications of the colorimetric test are given in Table VIII, the value for the poorest oil L being taken as unity.

Table VIII.

Oil	Relative intensities of blue colours measured by Lovibond tintometer	Relative intensities of blue colours measured by spectrophotometer
C	2·5	2·65
F	3·3	3·30
G	3·0	3·14
J	1·7	1·70
K	1·8	1·77
L	1·0	1·0

The agreement is satisfactory.

(*c*) Measurements of relative intensities at the maximum (328 $\mu\mu$) of the absorption band of cod-liver oils.

The intensity of absorption at 328 $\mu\mu$ can be measured to within about 2 % by the ordinary methods of quantitative spectrophotometry using the photographic technique, and to a definitely greater degree of accuracy by means of a photoelectric method. The former method was sufficiently accurate for our requirements, because a much larger source of uncertainty exists and has to be allowed for. The absorption spectrum of a material containing vitamin A is not as a rule due at 328 $\mu\mu$ to vitamin A solely, but the observed curve is made up of two super-imposed curves. If a number of samples of cod-liver oil are being compared, it is reasonable to suppose that the fatty acid glycerides and other normal oil constituents will generally absorb to approximately the same extent.

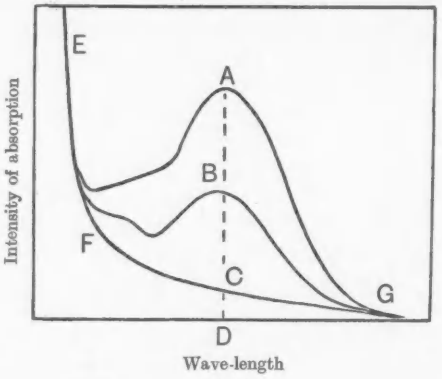

Fig. 1.

The curves in Fig. 1 represent diagrammatically the absorption of two different samples of cod-liver oil. *EFCG* represents the most probable curve for a typical cod-liver oil minus vitamin A, so that the relative intensities of the "vitamin" band are given by *AC* and *BC*. In actual practice, the distance *CD* has been determined from the mean of a large number of curves obtained with spent oils from which the vitamin has been extracted, very poor cod-liver oils, and oils in which the vitamin had been destroyed by photochemical or chemical means. It must, however, be pointed out that the measurements on the absorption band at 328 $\mu\mu$ lose some of their quantitative value when the persistence of the band is small, but gain very much in accuracy as the persistence of the band increases.

The values obtained in the examination of the six cod-liver oils are given in Table IX.

Table IX. *Spectrographic examination of oils.*

1 % solutions in alcohol, cell thickness 1 cm.

Oil	*AD*	Correction *CD* minus	Persistence of the ultra-violet band alone	Relative intensity L = unity
C	0·96	0·22	0·74	2·65
F	1·13	0·22	0·91	3·25
G	1·13	0·22	0·91	3·25
J	0·62	0·22	0·40	1·5
K	0·66	0·22	0·44	1·7
L	0·50	0·22	0·28	1·0

The curves for the six oils are given, together with those obtained on samples of the oils after 12 months storage, in Figs. 2, 3 and 4.

We are now in a position to assemble our results.

Table X. *Comparison of biological and physical measurements of the relative vitamin A value of the six cod-liver oils.*

Oil	Biological method	Colorimetric method		Ultra-violet band
		Tintometer	Spectrometer	
C	2	2·5	2·65	2·65
F	5	3·3	3·3	3·25
G	>2	3·0	3·15	3·25
J	2	1·7	1·7	1·5
K	1·5	1·8	1·77	1·7
L	1	1·0	1·0	1·0

All things considered, the agreement is remarkably good, particularly when the weaknesses of the biological method are borne in mind.

Examination of the six cod-liver oils after a year's storage. The six oils that were studied in the main part of this work were re-examined after they had been stored for just over a year. Two samples of each had been kept, one preserved in the dark, the other exposed to the daylight of a London laboratory. Both samples were contained in white glass bottles of 250 cc. capacity, closed with ground glass stoppers, and with an air space of about 20 cc. The examination by the biological test was not conducted as thoroughly as in the original tests. Four samples only were tested, and the animal groups were not sufficiently large. The results of the colorimetric and spectrographic tests are given in Table XI. The curves representing the latter are given in Figs. 2, 3 and 4.

Biological tests were made on oils C_1, C_2, L_1 and L_2. These represent the original oils C and L after storage for a year in light and dark respectively. The results are given in Table XII.

To attempt to estimate from these animal experiments—which were after all as complete a test of the vitamin A content as is often made—to what extent the vitamin has been destroyed during storage is almost impossible. The most that can be said is that destruction has proceeded further in the samples stored in light than in those kept in the dark, as would be expected,

and that in the former it is probably of the order of 50–60 % of the active substance originally present.

Doubtless a more thorough biological examination would have provided slightly more reliable evidence, but there is even in this admittedly superficial examination a general agreement between the results of the animal tests and the two physical methods of measurement.

Experiments on other materials. In the Medical Research Council Committee's report [1928] stress was laid on the importance of testing the colorimetric method on other products than cod-liver oil before drawing conclusions as to its general validity.

Although we have not investigated oils of plant origin, as was there suggested, we have submitted a variety of products to careful biological test and to examination by the two physical methods. The results are presented in Table XIII.

Table XI. *Ageing of cod-liver oils.*

Oil (1)	Date of examination (2)	Condition of storage (3)	Log I_0/I at 328 $\mu\mu$, 1 % solution 1 cm. cell (4)	Persistence of band × 100 (correction)* 0·13 (5)	Persistence of band × 100 (correction)* 0·22 (6)	Antimony trichloride blue: Log I_0/I at 608 $\mu\mu$, 1 % oil in 30 % $SbCl_3$, 2 cm. cell (7)	Antimony trichloride blue: Relative intensity blue colour (8)	Percentage loss (averages from figures in columns 5 and 8): In light	Percentage loss (averages from figures in columns 5 and 8): In dark
C	C Aug. 1928	—	0·96	83	74	2·75	83		
	C_1 May 1929	Light	0·54	41	32	1·41	42	50	
	C_2 May 1929	Dark	0·73	60	51	1·96	59		28·3
F	F Aug. 1928	—	1·13	100	91	3·3	100		
	F_1 May 1929	Light	0·83	70	61	1·8	55	37·5	
	F_2 May 1929	Dark	0·95	82	73	2·25	68		25
G	G June 1928	—	1·13	100	91	3·11	94		
	G_1 May 1929	Light	0·82	69	60	1·84	56	35·5	
	G_2 May 1929	Dark	1·05	92	83	2·25	68		17·5
J	J Aug. 1928	—	0·62	49	40	1·85	56		
	J_1 May 1929	Light	0·36	23	13	0·8	24	55·2	
	J_2 May 1929	Dark	0·53	40	31	1·2	36		27·7
K	K June 1928	—	0·66	53	44	1·76	53		
	K_1 May 1929	Light	0·39	26	17	1·04	31	46·2	
	K_2 May 1929	Dark	0·51	38	29	1·28	39		27·4
L	L June 1928	—	0·50	37	28	1·0	30		
	L_1 May 1929	Light	0·21	8	—	0·4	12	70	
	L_2 May 1929	Dark	0·39	26	17	0·84	25		24
							Av.	49	25

* 0·22 seemed to be the most reasonable allowance to make for the absorption of the fresh oils at 328 $\mu\mu$ in respect of constituents other than vitamin A. This appears to be rather too high for the oils after standing. The differences may quite possibly be connected with the vitamin D potency. Both corrections have been inserted for all the oils, as the two figures for each oil demonstrate the real limitations of the method.

The agreement between the results obtained with the three tests is on the whole satisfactory, and strengthens confidence in the colorimetric and spectrographic methods of assay. Either vitamin A itself shows selective ultra-violet absorption from 280–360 $\mu\mu$ and is responsible for the blue colour,

Table XII.

Oil	Dose mg.	No. of rats in group	Average rate of growth g. weekly	Estimated loss due to storage	Estimated percentage loss detected by physical methods Table XI
C	5	6	2·8	—	—
C_1 (light)	5	4	1·0	About 50 %	50
	10	4	2·9		
C_2 (dark)	5	4	1·6	Probably less than 50 %	28
	10	3	2·9		
L	10	6	2·0	—	—
L_1 (light)	10	3	−0·6	More than 50 %, probably nearer 70 %	70
	20	3	1·0		
	30	3	2·2		
L_2 (dark)	10	3	−0·2	More than 50 %	24
	20	4	1·9		

Table XIII.

Material tested	Biological test ("recovery" test) Dose mg.	Result	Colorimetric test	Spectrographic test
(1) Caplin oil (oil extracted from whole body)	10	No growth	No colour	No band
	20	No growth		
(2) Squid (body oil)	20	Very slight growth	Slight transient blue colour, head of band about 598 $\mu\mu$	Small inflexion 306–336 $\mu\mu$
	40	Growth about 5 g. weekly		
3) Oil extracted from *Nephrops norvegicus*	30	No growth	No colour	No band with max. near 328 $\mu\mu$
	60	No growth		
(4) Oil extracted from *Meganyctiphanes norvegicus*	30	No growth	Little if any colour	Small inflexion near 320 $\mu\mu$
	60	Very slight growth		
(5) Oil extracted from *Calanus finmarchicus*	30	No growth	No colour	
	60	No growth		
(6) Unsaponifiable fraction from sample of plankton oil (5)	30	No growth	No colour	
	60	No growth		
(7) Oil extracted from a mixed plankton sample consisting largely of copepods	30	No growth	No colour	
	10	No growth		
(8) Oil extracted from sheep liver	0·1	Growth about 3–5 g. weekly	About 14 times as rich as cod-liver oil "G" by both methods	
	0·3	Growth about 8 g. weekly		
(9) Unsaponifiable fraction from sample of sheep-liver oil (8)	0·005	Growth 2–3 g. weekly	About 9 times (8)	About 12 times (8)
	0·01	Growth 5–7 g. weekly		
	0·03	Growth 10 g. weekly		

with an absorption maximum near 608 $\mu\mu$, in the antimony trichloride test, or else these are properties of other substances contained in cod-liver oil and exhibiting a remarkable similarity to the vitamin as regards distribution and behaviour.

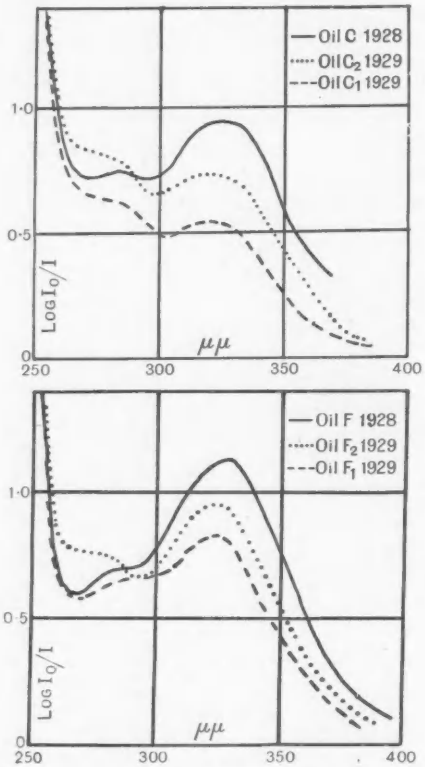

Fig. 2.

Objections to the specificity of the colour test for vitamin A. A number of papers have appeared recently in which the value of the colour test as an indication of the presence of vitamin A has been questioned. The relation of this test to the colour reactions of carotene and certain other substances is discussed in another paper [Dulière, Morton and Drummond, 1929] and is not directly related to the subject of this section.

Schmidt-Nielsen [1928] states that samples of herring oil gave vitamin A values of 50–180 units when tested by the U.S. Pharmacopœia method, but failed to give the blue colour with antimony or arsenic trichloride.

It is also stated that the oils of the flesh of sprat and mackerel are good sources of vitamin A, but do not give the blue colour test, and that the oil of the caplin, whilst failing to respond to the test, "ought to be rather rich" in vitamin A. We have examined three samples of caplin oil and have found them uniformly inactive in doses of 10 and 20 mg.

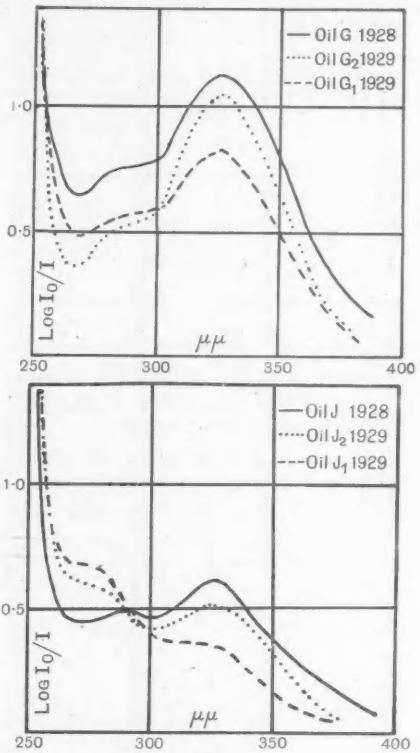

Fig. 3.

No details are given in these papers of the animal experiments beyond the statement that vitamin D was supplied, so that it cannot be said whether they were in all respects satisfactory, particularly as regards the length of the test and the number of animals employed. At present we are unable to explain the results of Schmidt-Nielsen. We are preparing for examination a number of body-oils of fish similar to those used by him[1]. In addition, we hope to obtain for comparative tests samples of the oils actually examined by him.

[1] Dr Ahmad, working in the laboratory at University College, is making this examination with salmon-body oil and salmon-liver oil. The results of the biological test so far are in agreement with the colorimetric test.

The comparative examinations of cod-liver oils by the biological and colorimetric methods recorded by Bailey, Cannon and Fisher [1928] and by Jones, Briod, Arzoomanian and Christiansen [1929] appear to be without significance, as the biological method employed was that recommended in the 1926 edition of the U.S. Pharmacopœia. It has been repeatedly pointed out that this method is liable to give highly misleading results, owing to the absence of vitamin D during the depletion period.

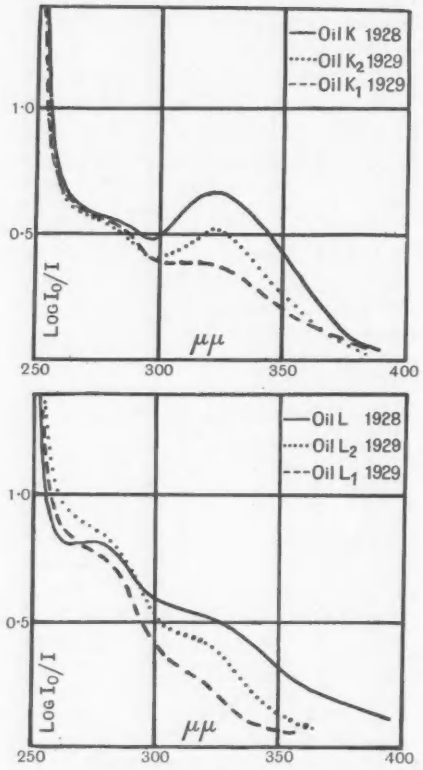

Fig. 4.

The adverse criticisms of Steudel [1927, 1929] regarding the specificity of the blue colour reaction do not rest on a firm basis. From the growth curves given in his paper it is apparent that the vitamin A "preparations" with which he was working were of so low an activity that from 1 to 2 g. were required daily to promote a rather poor rate of growth in rats. It is not to be expected that the colour reaction would be exhibited except very faintly by a material so poor in vitamin A. The colour reaction, even using the

more sensitive arsenic trichloride reagent, is given but feebly by samples of butter that promote growth in daily doses of from 0·1 to 0·4 g. In investigating such materials it is necessary, before applying the colour test, to concentrate the chromogenic substance by preparing the unsaponifiable fraction.

We have found it difficult to apply the colour test to the estimation of vitamin A in butter because of the similar blue colours produced by the lipochrome pigments. No entirely satisfactory method of removing these pigments or of making an allowance for the blue colour produced by them has yet been evolved. It is possible that determinations of the intensity of the 328 $\mu\mu$ band will provide a simple way out of the difficulty. Quite recently, however, Andersen and Nightingale [1929] have reported success in applying the colorimetric method to margarines containing vitamin A. The test was applied to the unsaponifiable matter and the results showed general agreement with those of the biological test.

It is also necessary to bear in mind that the colour reaction is inhibited by water and the lower alcohols.

Further work is needed to establish definitely whether the colour reaction and the absorption band at 328 $\mu\mu$ are in reality due to vitamin A, or whether they are caused by one or more than one substance closely resembling the vitamin in distribution and properties. A final decision on this point will not be reached until the substances concerned have been isolated. The absorption band at 328 $\mu\mu$ by itself is probably not a reliable index of vitamin A as such [Rosenheim and Webster, 1929], but we are satisfied that for cod-liver oils the colour test and the measurement of the intensity of the 328 $\mu\mu$ band taken together are more reliable indications of the relative vitamin A potencies than the biological tests, unless the latter are performed on an unusually large number of animals.

Summary.

1. Six samples of cod-liver oil have been examined for vitamin A by the biological method. In spite of the use of much larger groups of rats (7–12) for each dose than are usually employed, the individual responses of the animals showed such wide variations as to make it a very difficult matter to detect quantitative differences in vitamin A potency of less than 100 %.

2. As far as the relative values of the six oils could be estimated by the biological test, they were in agreement with the response to the colour reaction of Rosenheim and Drummond, and with the relative intensities of the absorption band (328 $\mu\mu$) believed by Morton and Heilbron to be characteristic of vitamin A. The agreement between the results of the colorimetric and spectrographic methods was very close.

3. A comparison of the biological assay with the two physical methods was also made in the case of a number of other oils, and general agreement between the results found.

4. In view of the statement of other investigators that certain oils (fish-body oils) may be rich in vitamin A without showing the colour reaction, it seems still undesirable to claim that the colorimetric method can generally replace the tedious and inaccurate biological tests. Actually, in a wide experience, we have not encountered a single instance in which there has been disagreement between the animal tests and the intensity of the blue colour showing maximum absorption near 608 $\mu\mu$. As far as cod-liver oils are concerned we have no hesitation in recommending the colorimetric and spectrographic methods as giving rapid, and reliable, quantitative results.

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LXXXIX. VITAMIN A AND CAROTENE.

I. THE ASSOCIATION OF VITAMIN A ACTIVITY WITH CAROTENE IN THE CARROT ROOT.

By THOMAS MOORE.

(*Report to the Medical Research Council.*)

From the Nutritional Laboratory, Cambridge.

(*Received July 5th, 1929.*)

The possibility that vitamin A activity may bear some relation to carotenoid pigmentation has attracted the attention of many workers during the past ten years. Of early investigations those of Steenbock and his colleagues [Steenbock, Boutwell and Kent, 1920; Steenbock, 1919; Steenbock and Gross, 1919, 1920; Steenbock and Boutwell, 1920, 1, 2; Steenbock, Sell and Boutwell, 1921; Steenbock and Sell, 1922] were perhaps the most extensive, and furnished a weight of evidence pointing to some connection between yellow pigmentation and vitamin A activity at least in so far as concerned vegetable sources. This connection was confirmed somewhat later by Coward [1923], who found that "some lipochrome (generally carotene) is always associated with the vitamin in plant tissues; and that where carotene is found, particularly carotene exposed to sunlight, there the vitamin can be expected to be present also."

Rosenheim and Drummond [1920], although denying that the vitamin could be either carotene or xanthophyll, were attracted by the possibility of a relationship with some unknown pigment, and suggested a parallelism between yellow coloration and vitamin A activity even in animal sources. In this direction, however, it soon became obvious that no relationship existed [Drummond and Coward, 1920]. It was true that yellow butter fat was an active source whereas colourless lard was inactive, but Stephenson [1920] found that butter fat could be completely decolorised by charcoal without losing its activity. Similarly in the case of cod-liver oil, found by Zilva and Miura [1921] to be an extremely rich source of the vitamin, pigmentation was valueless as a guide to activity; pale cod-liver oils displayed much greater activity than highly pigmented butter fat. Working upon different lines Palmer and his colleagues, who had been the first to prove that the lipochrome pigments of animals are derived from the plant pigments present in their food [Palmer and Eccles, 1914], achieved success in rearing chickens and rats upon diets almost devoid of carotenoids [Palmer and Kempster, 1919; Palmer and Kennedy, 1921].

The above indirect evidence, which seemed to rule out the possibility that vitamin A might be a pigmented body, was supplemented from time to time by direct tests upon isolated pigments. Steenbock, Sell, Nelson and Buell [1921] made a preliminary announcement that carotene, even after several recrystallisations, possessed the activity of vitamin A, but no detailed account of this work seems to have been published. Drummond [1919], on the other hand, found that while impure carotene showed signs of activity in the rat at doses of about 0·3 mg. daily, similar doses of the pure pigment were quite ineffective. Stephenson [1920] tested carotene recrystallised four times and obtained a similar negative result. Subsequently Drummond, Channon and Coward [1925], dissatisfied that the activity of cod-liver oil concentrates might not after all be due to traces of carotenoids, carried out further tests, finding both carotene and lycopin to be inactive. A similar result was obtained in the case of xanthophyll by Willimott and Moore [1927]. Underhill and Mendel [1928], on the other hand, found carotene to possess physiological activity of a kind, since it was found to be highly effective in curing "black tongue" disease in dogs.

In the face of this accumulation of evidence it was not unnatural that the idea of the identity of vitamin A with a carotenoid pigment became generally discredited. Even at an early date Steenbock, Sell and Buell [1921] had been forced to admit that, unless the carotenoids could exist in leuco form, "their coincident occurrence in nature might be due to physiological determination."

The work of Euler, Euler and Hellstrøm [1928], however, has reopened the whole question of the vitamin A activity of the carotenoids. These workers pointed out that in previous biological tests of carotene no provision was made for the presence of vitamin D in the diet, and that the results obtained cannot therefore have been reliable. Attracted by similarities between the colour reactions given by the vitamin and by certain carotenoids with the antimony trichloride reagent [Carr and Price, 1926], they put carotene to further physiological tests, with the result that they found it to be active in doses of 0·005 mg. per rat daily.

It may be said that the general resemblance of the colour reactions of the vitamin to those of the carotenoids had been already familiar [Rosenheim and Drummond, 1920, 1925; Takahashi *et al.*, 1925] and the main interest of the new work obviously rested in the claim that chemically pure carotene could restore growth in rats suffering from vitamin A deficiency. In view of divergent spectroscopic data it was considered improbable by the Swedish workers that the vitamin A of cod-liver oil was carotene itself, and the suggestion was put forward that several substances possessing the physiological activity of the vitamin might exist. The vitamin A of cod-liver oil, for example, might well be derived from the carotenoids of marine algae. In a later communication [Karrer, Euler and Euler, 1928] the suggestion has been made that vitamin A activity is not the property of any single substance, but is due to the presence in various molecules of the "polyene" grouping, a

system of double bonds which gives rise to colour reactions and which is held to possess catalytic powers *in vivo*.

Preliminary experiments by the writer in confirmation of the activity of carotene have been mentioned elsewhere [Moore, 1929]. Two samples of the pigment, kindly made available by Prof. von Euler and Dr R. A. Morton, were both found to restore good growth in rats in doses of 0·01 mg. daily. Dulière, Morton and Drummond [1929] on the other hand, after preparing an exceptionally pure sample of carotene (M.P. 184·9°), have obtained no evidence of growth-promoting power, and have suggested that the specimens used by Euler and his colleagues were probably contaminated with traces of the vitamin carried down as impurity.

In the present communication a study of the vitamin A activity of the carrot root will be described. It will be shown that this material provides a much richer source of vitamin A activity than has hitherto been supposed, and that activity tends to be concentrated in the carotene during its isolation, remaining in the pigment through all stages of purification that have so far been applied. This finding is in agreement with the conclusion of Collison, Hume, Smedley-MacLean and Smith [1929] that carotene isolated from cabbage fat also possesses intense vitamin A activity.

Experimental.

In testing the vitamin A activity of the various materials the following basal diet was used:

Casein (Glaxo "Vitamin Free")	20 %
Rice starch	60 %
Arachis oil	15 %
Salt mixture	5 %

The vitamin B complex was supplied by the addition of 7·5 % of a standard marmite extract; vitamin D by an allowance of one drop of "Radiostol" (irradiated ergosterol in arachis oil) per animal daily.

Young albino rats receiving this diet at first continued to grow for varying periods, but in the end invariably declined and died. During decline signs of xerophthalmia and of the characteristic lung troubles were frequently in evidence. Growth curves of control rats are shown in Fig. 1[1].

The vitamin A activity of fresh carrot. Calculations based upon the reported activity of carotene led to the obvious implication that if the claim was valid the carrot must be a much richer source of vitamin A activity than hitherto supposed. That this is actually the case has been shown in the following experiments.

[1] It will be noticed that the maximum weights attained by the rats varied within wide limits. Such differences were probably attributable in part to differences in vitamin A reserves between various litters and in part to variations in the initial weights at which the animals first received the experimental diet. The degree of variation in the negative control animals was emphasised by the selection for this purpose of certain rats which attained unduly high maximum weights.

The test rats were restricted to the basal diet until definite signs of vitamin A deficiency had become obvious, whereupon fresh carrot root, cut into pieces of 50, 100 or 200 mg., was presented daily before the basal ration.

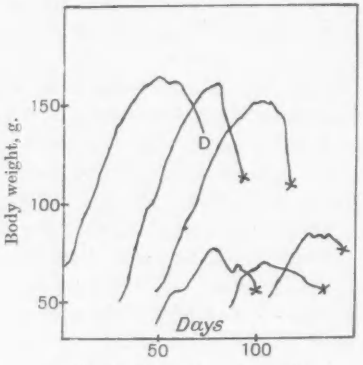

Fig. 1. Negative control rats.
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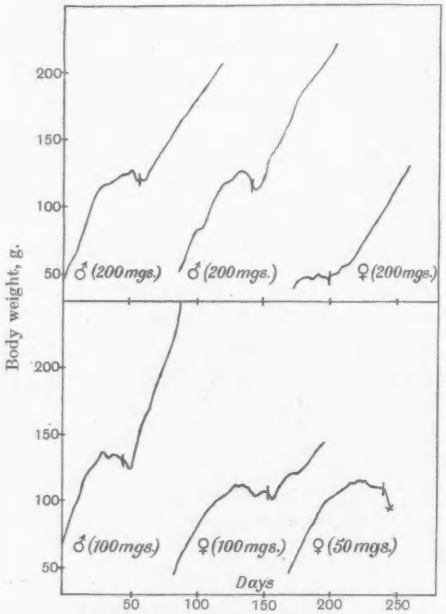

Fig. 2. Rats receiving fresh carrot.

The responses obtained are shown in Fig. 2. In all rats receiving 200 or 100 mg. of carrot xerophthalmia was cured and steady growth was resumed

until the discontinuation of the experiment. In one rat receiving 50 mg. of carrot no response was obtained. It would therefore appear that the allowance of fresh carrot necessary to supply the rat's requirement for vitamin A is about 100 mg., although it is unsafe to assume from the evidence of a single animal that a slightly lower dose might not sometimes be effective.

The vitamin A activity of carrot fat. The fresh carrot is said to contain 0·6 % of fat. If we assume that all the vitamin A activity of the root is removed with the fat during the extraction with light petroleum it is obvious that 100 mg. of fresh carrot must be represented by 0·6 mg. of the fatty residues obtained after the evaporation of the light petroleum.

In a trial experiment (prior to the extraction of the pigment on a larger scale) about 75 kg. of carrots were first sliced and dried in a hot room, and were then forwarded to Messrs British Drug Houses, Ltd., who kindly carried out the extraction. After grinding to a fine powder the material (now amounting to 7·5 kg.) was extracted repeatedly with light petroleum, which was finally removed under diminished pressure.

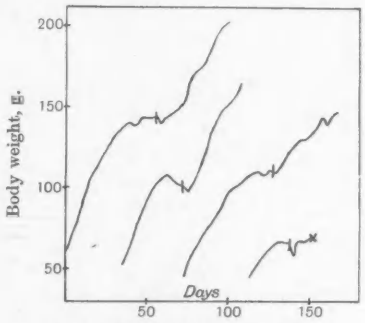

Fig. 3. Rats receiving carrot fat.

On addition of excess of methyl alcohol to the extract so obtained a yield of crude carotene amounting to about 2 g. was precipitated. The unprecipitable fraction, a deep crimson fat of low melting-point, amounted to some 45 g.

The main interest of the present experiments lay in the comparison of the vitamin A activity of carotene with that of the mother-liquor from which it had been separated. The biological test, therefore, was carried out upon the unprecipitable fraction rather than upon the original extract. The growth curves of four rats which received daily doses of 0·4 mg. of this material are shown in Fig. 3. In all four cases a resumption in growth was observed, but one animal died suddenly after receiving the fat for a short period.

Biological experiments at a lower dosage were not carried out. Colorimetric data, however, indicated that the activity of this fat was considerably inferior to that of crystalline carotene. The chromogenic value of the fat,

estimated by means of the antimony trichloride reagent, was found to be only 12 Lovibond blue units per mg. per cm. cube of reagent, as against 165 units per mg. of carotene tested under similar conditions. The blue coloration produced by the fat with the antimony trichloride reagent when inspected by means of a direct-vision spectroscope was found to be characterised by an absorption band at about 590 $\mu\mu$.

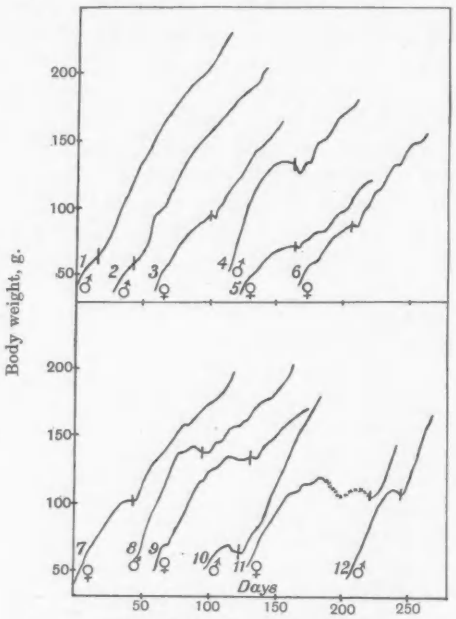

Fig. 4. Rats receiving carotene.

Rat No.	Dose of carotene
1, 2	Sample A. 0·02 mg.
3, 4, 5	Sample B. 0·01 mg.
6, 7, 8, 9, 10	Sample C. 0·01 mg.
11, 12	Sample D. 0·01 mg.

××××× Rat receiving inactive substance.

The vitamin A activity of carotene. Four samples of carotene in all have been tested. Of these the samples A and B, both specimens of a fair degree of purity, were made available through the kindness of Prof. von Euler and of Dr R. A. Morton. Sample C (M.P. 174°) was prepared in Karrer's laboratory by submitting a sample already tolerably pure (M.P. 169°) to nine further crystallisations from light petroleum. Sample D (M.P. 174° in air) was prepared by the writer from a specimen (M.P. 174° in air[1]) already purified to a

[1] The melting-points of carotene samples vary considerably according to the technique employed. The melting-point of this specimen, for example, was found by Prof. Drummond, to whom my best thanks are due, to be about 180° when heating was carried out rapidly in an atmosphere of nitrogen.

high degree by many crystallisations from methyl-alcohol-chloroform mixtures, by submitting it to a further five crystallisations from cyclohexane.

In order to facilitate administration the samples to be tested were dissolved in warm arachis oil in such concentration that standardised drops of the oil (20 mg.) contained 0·01 mg. of carotene. The growth responses obtained by administering the pigment daily to rats deficient in vitamin A are shown in Fig. 4. The activity of all the samples at this low level was unquestionable. Xerophthalmia was invariably cured and good growth restored over prolonged periods[1].

Spectroscopic examination of the blue colour produced by samples of carotene when treated with antimony trichloride showed a marked band at about 590 $\mu\mu$, *i.e.* the position described by Euler, Euler and Hellstrøm, and confirmed by Dulière, Morton and Drummond. No trace of absorption was visible at 610 $\mu\mu$, the position considered characteristic of the cod-liver oil vitamin A.

Discussion.

The above results confirm the claim of Euler, Euler and Hellstrøm that carotene possesses the physiological action of vitamin A. The suggestion of Dulière, Morton and Drummond that pure carotene is inactive, and that the activity reported by the Swedish workers may have been due merely to contaminating traces of vitamin A, must be considered improbable for the following reasons. (1) Carotene samples of a high degree of purity were found invariably to be active. (2) The activity of the carrot fat from which the carotene was crystallised was inferior to that of the isolated pigment. The activity of the pigment therefore could not have been due to the presence of fat carried down as impurity. (3) Both carotene and carrot fat produce with antimony trichloride a blue coloration which is characterised by an absorption band at 590 $\mu\mu$. If the physiological activity of these sources were attributable to the same vitamin A as contained in cod-liver oil, intense absorption would be anticipated at 610 $\mu\mu$, but no band was visible in this position.

The possibility of the presence of an uncharacterised active impurity, even in the purest samples of carotene so far obtained, cannot of course be ruled out. Neither can we be sure that carotene samples, even if pure, do not tend to change with the formation of an active product during the processes involved in the administration of test doses to rats. It seems highly probable, however, that the samples found active in the present experiments differed but little in regard to purity from those found inactive by Drummond and

[1] In order to decide whether the addition of vitamin D to the test diet was really essential in maintaining growth, two rats (Sample A) were deprived of the customary allowance of "Radiostol," but were given carotene (0·02 mg.) almost from the start of the experiment. Under these conditions no check in growth was observed. Whether growth may continue in the absence of vitamin D (as suggested by Euler, Rydbom and Hellstrøm, 1929) or whether the present test diet was not free from traces of this vitamin cannot yet be decided. A direct test of the vitamin D content of carotene is in progress.

his colleagues, and that the main cause of such completely divergent results may well be found in some unsuspected difference in biological technique. It may be pointed out that, whereas those workers who have employed basal diets containing fat have obtained positive results, Drummond and his colleagues, presumably using the standardised "fat-free" diet of their laboratory, have obtained a negative result.

While there can be no doubt as to the activity of carotene under the experimental conditions described, it remains to be explained how molecules, differing so obviously as do carotene and the vitamin A of cod-liver oil, can be interchangeable in function. The "polyene" theory of Euler and his colleagues is open to the criticism that a very low order of specificity, quite unlike that of vitamin D, must be implied. Preliminary experiments by the writer, on the other hand, have indicated that a chemical relationship between carotene and vitamin A may exist. It is hoped to make this question the subject of a later communication.

The high vitamin A activity of the carrot may be of interest from an economic standpoint. The minimum dosage of 100 mg. found effective in the present experiments contained only 10 mg. of dry matter, and represented only one-tenth of the dosage previously considered necessary [Osborne and Mendel, 1920].

Summary.

1. Under the experimental conditions specified, fresh carrot root was found to be a much richer source of vitamin A activity than hitherto supposed. Daily doses of 100 mg. of the fresh root sufficed to cure xerophthalmia and restore good growth in rats deprived of vitamin A.

2. Under similar conditions a sample of carrot fat, from which much of the carotene had been removed, was found to be active in a dose of 0·4 mg. daily.

3. Carotene (M.P. 174° in air) was found, even after many recrystallisations, to be active in doses of 0·01 mg.

4. A possible explanation of the divergent results of other workers is put forward.

My special thanks are due to Dr L. J. Harris for helpful criticism. I am also very much indebted to Messrs Chivers and Sons, Ltd., and to Messrs British Drug Houses, Ltd., for their willing co-operation in preparing large amounts of carotene. The care of the experimental animals was in the capable hands of Mr A. Ward.

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XC. THE EFFECT OF CYANIDE ON THE RESPIRATION OF ANIMAL TISSUES.

By MALCOLM DIXON and KENNETH ALLAN CALDWELL ELLIOTT.

From the Biochemical Laboratory, Cambridge.

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According to Warburg's theory of cell respiration the absorption of oxygen by tissues is due entirely to the reaction between molecular oxygen and a complex intracellular iron compound. This iron compound, which Warburg believes to be a haematin derivative, he has termed "the respiratory enzyme" (das Atmungsferment), and he considers that molecular oxygen brings about oxidations in the tissues only by its action. "Die Primärreaktion der Atmung ist die Reaktion zwischen molekularem Sauerstoff und Eisen, und nur in dieser Reaktion, nicht mit den organischen Molekülen, vermag der molekulare Sauerstoff in der Zelle zu reagieren" [Warburg, 1923, 1]. According to this theory, then, tissue respiration is to be regarded as being due to the action of one catalytic iron-containing system, rather than as the sum-total of a number of oxidations brought about by a number of more or less independent catalysts.

The theory is based principally on measurements of the inhibiting effect of cyanides, carbon monoxide and similar substances on cell respiration. It is well known that such substances act as powerful inhibitors of most iron-catalysed reactions, and the marked inhibition of cell respiration which they produce is considered to be due to a poisoning of the "respiratory enzyme."

It was pointed out by Dixon and Thurlow [1925] that animal tissues contain systems, such as the xanthine oxidase, whose oxygen uptake is not affected by cyanide, carbon monoxide, etc., and they considered that such systems formed exceptions to Warburg's general statement. The essential part of Warburg's reply was as follows [Warburg, 1925]: "Nach der Theorie sind diejenigen Lebensvorgänge, die durch Blausäure spezifisch gehemmt werden, Schwermetallkatalysen, so die Leberatmung, die durch Blausäure spezifisch gehemmt wird. Wenn die Oxydation von Hypoxanthin durch Blausäure *nicht* gehemmt wird, so liegt hier ein System vor, das in der Atmung der Leber keine Rolle spielt, ein System, das *kein* Respirationssystem ist." In other words: cell respiration is inhibited by HCN, xanthine oxidase is not, therefore xanthine oxidase is not a cell respiration system[1].

Such an argument is obviously valid only if cyanide produces a total inhibition of tissue respiration; if the inhibition is only partial, such systems as the xanthine oxidase may well contribute to that part of the respiration which is not inhibited by cyanide.

[1] It is difficult to see why xanthine oxidase should not be classed as a respiration system, since it is present in the tissues in an active form and the oxidation it brings about is known to occur in the tissues.

Warburg [1928] has recently further defined his position as follows: "Xanthinoxydase und Succinoxydase sind Niederschläge aus Zellextrakten[1], die weder wie das Atmungsferment wirken, noch wie das Atmungsferment beeinflusst werden. Vielleicht enthalten sie denaturierte Reste von Atmungsferment. Nennt man diese Niederschläge Atmungsfermente, so hat man zu unterscheiden zwischen Atmungsfermenten, die die Erscheinungen der Atmung hervorrufen, und solchen, die es nicht tun." This again is only valid so long as the whole of the respiration is due to systems inhibited by cyanide. Warburg continues "Beschränken wir uns auf die Fermente erster Art, so ist auf *Dixons* Frage, wieviel Atmungsfermente es gibt, folgendes zu antworten: Jeder weiss, dass sich die Natur zum Sauerstofftransport vorwiegend des Hämoglobins bedient, dass nicht alle Hämoglobine identisch sind und dass auch andere Sauerstofftransportmittel, z.B. Hämocyanin, existieren. Entsprechend gibt es in der Natur nicht nur 1 Atmungsferment, aber der Mechanismus der Atmung in einem bestimmten Falle ist, wie der Mechanismus des Sauerstofftransports, ein bestimmter und einheitlich. Wie zum Transport des Sauerstoffs, so benutzt die Natur zur Verbrennung durch Sauerstoff *vorwiegend einen* Mechanismus. Denn das Atmungsferment in Hefezellen und Chorion, in Leberzellen und Blutplättchen, in Leucocyten und Seeigeleiern verhält sich im Prinzip gleich."

In view therefore of what is based on the assumption that the *whole* of the respiration of animal tissues is inhibited by cyanide it is necessary to enquire upon what experimental evidence it rests. It appears that practically all the measurements of the effect of cyanide on cell respiration upon which Warburg's theory is based were carried out on such cells as yeast cells, sea urchins' eggs, red blood-corpuscles, etc. Very few measurements appear to have been done on the more typical animal tissues, such as liver, muscle, etc. In a fairly recent paper Warburg [1926] states that the respiration of animal tissues is almost completely inhibited by hydrogen cyanide, giving a reference to an earlier paper [Warburg, Posener and Negelein, 1924]. In this earlier paper the same statement is made, but is supported by only a single experiment, which was carried out on embryonic tissue, and which showed an inhibition of 60 % with $M/1000$ HCN. A search of Warburg's papers revealed one other such experiment [Warburg, 1926, p. 438], showing a 90 % inhibition with kidney and $M/1000$ HCN. So far as we are aware, Warburg has not published any other experiments on the effect of cyanide on typical animal tissues. Meyerhof [1923] found that the respiration of frog muscle was completely inhibited by $M/200$ cyanide. Batelli and Stern [1907] performed some experiments on a few animal tissues which usually gave a very incomplete inhibition, but in one or two cases showed an almost complete inhibition

[1] Incidentally we would point out that xanthine oxidase and succinoxidase, far from being mere precipitates of cell extracts, are two of the most sharply defined and specific enzymes known. They can be prepared by a variety of methods, or studied in the tissue itself. Their behaviour is the same in either case.

when a high concentration of cyanide was used. Few experimental details are given, however, and they do not state that the cyanide was neutralised before use. Neglect of this precaution has vitiated experiments carried out by other authors in some cases. Resnitschenko [1928] gives a number of experiments on *Ascaris* eggs, pointing out later that much of the effect was due to the alkalinity of the cyanide rather than to a specific poisoning. We may mention here the experiments of Allen [1919], who found that cyanide was capable of inhibiting 80 % of the respiration of *Planaria*.

The scarcity of data on animal tissues, coupled with the facts mentioned above, made it highly desirable that a systematic study of the degree of inhibition produced by cyanide on the respiration of a number of typical animal tissues should be carried out. The results of such an investigation are described in the present paper.

The technique employed was as follows, except where otherwise stated. Four differential Barcroft micro-respirometers were used in each experiment. Into the right-hand flask of each apparatus was weighed 0·5 g. of the tissue (the preparation of which is described below), and 1·5 cc. of phosphate buffer p_H 7·3 added. To the four flasks were also added 1 cc. of water, $M/1000$ KCN, $M/100$ KCN, and $M/10$ KCN respectively. The four left-hand (compensation) flasks contained the same cyanide solutions as the corresponding right-hand flasks, together with 2 cc. of phosphate buffer. The total volume added to each flask was therefore 3 cc., so that the final concentrations of cyanide in the four respirometers were 0, $M/3000$, $M/300$, $M/30$ respectively. The cyanide solutions were always carefully neutralised to p_H 7·3 before use. The small tubes in all the eight flasks contained rolls of filter-paper moistened with 6 % KOH so as to give efficient absorption of the CO_2 produced in respiration. The respirometers were then placed in the thermostat at 40° and shaken at a speed of about 120 complete oscillations per minute. After allowing about 5 minutes for equilibration, the taps of the apparatus were closed and readings commenced.

The tissues were used as soon as possible after the death of the animal. It should be noted that they were normal tissues, not washed or extracted in any way. The only treatment they received was the slicing or mincing, according to the form in which they were used. We carried out a number of experiments using tissues sliced according to Warburg's [1923, 2] method, *i.e.* cut with a razor moistened with Ringer's solution into slices 0·5 mm. or less in thickness. This method of preparation is recommended by Warburg for work on tissue respiration, as it avoids injury to the tissue as far as possible, while allowing the oxygen free access. We have also used the tissue in the form of a fine suspension, obtained by the use of a special fine mincer. The tissue, on being passed through this machine, without the addition of any liquid, was reduced to a thick homogeneous paste which, when weighed out into the flasks, readily dispersed in the liquid to give a uniform suspension. The suspension, though fine, was not so fine as to involve excessive injury to

the tissue cells. In a series of comparative experiments we found that the two methods of preparation gave identical results. The rate of respiration and the degree of inhibition produced by cyanide were the same in both cases. (Some of these experiments are quoted in Table I below.) We also determined the effect of substituting mammalian Ringer's solution for the phosphate buffer, using both slices and suspensions of tissue, and we were unable to detect any difference. The greater number of our experiments was therefore carried out on tissue suspensions in phosphate buffer solution.

We found that the reproducibility of the results was so satisfactory as to give us considerable confidence in the method. That is to say, duplicate determinations carried out on different samples of the same tissue always agreed very closely. As examples we may refer to the experiments given in Figs. 7 and 12. Comparing different tissues, however, considerable differences in respiration rate were observed, even with corresponding tissues from different

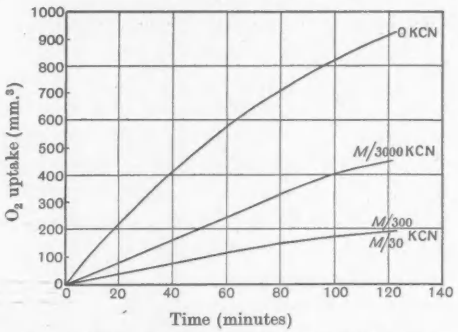

Fig. 1. Effect of cyanide on respiration of rabbit kidney.

individuals of the same species. This is not surprising, however, as differences of 2000 % are observed even by the most physiological method—that of perfusion [Bornstein and Gremels, 1928]. Probably variations in the state of nutrition have a considerable influence.

Considering now the results obtained with cyanide, four completely typical sets of experiments are represented in Figs. 1–4. It will be seen that the curves are practically linear, *i.e.* the oxygen uptake proceeds with a constant velocity, at any rate for the first hour. The cyanide produces a marked, but by no means complete, inhibition of respiration; usually it leaves about one-third of the total respiration unaffected. An important point is that with most animal tissues $M/300$ cyanide already produces the maximal inhibition obtainable, and this inhibition is not increased by increasing the cyanide concentration tenfold (or even a hundredfold, see below). This is characteristic of most of the animal tissues studied. Its significance is discussed later. These experiments clearly show that from one-half to one-quarter of the total respiration is due to systems which are not poisoned by cyanide.

The results obtained are more conveniently represented in the form of curves showing how the rate of respiration varies with the concentration of cyanide. The method of plotting these curves will be clear from Fig. 5, which also shows the typical form of the curve.

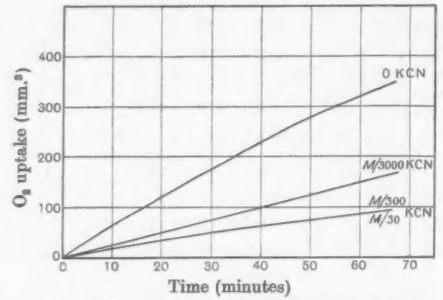

Fig. 2. Effect of cyanide on respiration of pig liver.

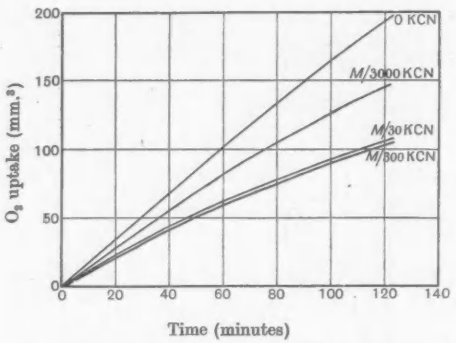

Fig. 3. Effect of cyanide on respiration of sheep heart.

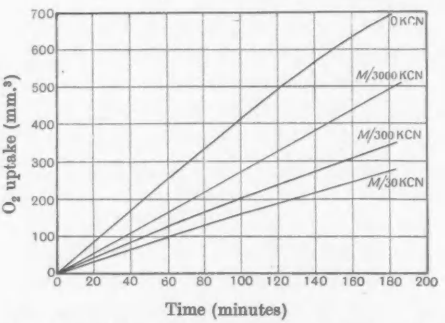

Fig. 4. Effect of cyanide on respiration of rat liver slices.

The results of a number of experiments on different tissues have been plotted in this form, and are represented in Fig. 6. The rates of respiration are here expressed as percentages of the corresponding rates with no cyanide,

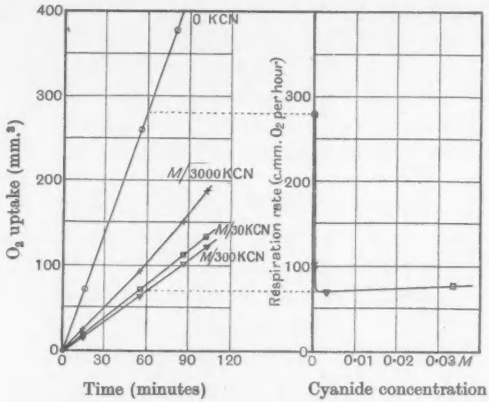

Fig. 5. Effect of cyanide on respiration of rabbit kidney slices, showing method of obtaining inhibition curves. In this experiment (No. 5 of Table I) the tissue slices had been kept in their respective cyanide solutions for 3 hours at 0°, so as to ensure thorough penetration of the cyanide.

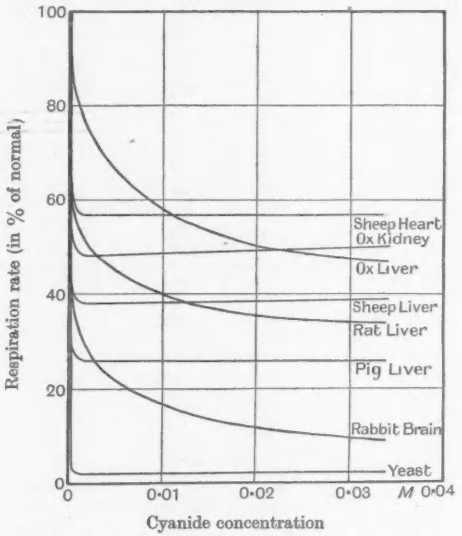

Fig. 6. Cyanide inhibition curves of various tissues.

in order to make the curves comparable. The curves are all of essentially the same form, but the concentration of cyanide necessary to produce maximal inhibition varies with different tissues, as also does the degree of inhibition

obtainable. In no animal tissue however is inhibition complete, however much cyanide is added.

In order to check our methods we carried out a number of experiments on yeast cells. Negelein [1925], working in Warburg's laboratory, has shown that cyanide produces a practically complete inhibition of the respiration of yeast, and our experiments entirely confirm his, showing usually a 95–98 % inhibition, as shown in the lowest curve in Fig. 6. It is clear that yeast behaves unlike animal tissues. We have, however, had one or two samples of yeast in which the inhibition was somewhat less.

It is obviously neither possible nor necessary to give the oxygen uptake curves, or even the inhibition curves, of a large number of experiments; and we have condensed the majority of our experiments into the form of a table showing the normal respiration rate and the percentage inhibition produced by various concentrations of cyanide for a number of different tissues. It should be understood that each figure in the table represents a separate oxygen uptake curve.

Table I.

Exp. No.	Tissue	Rate of O_2 uptake without KCN (mm.³ per g. per hr.)	Inhibition produced by (in % of normal) *M*/3000 KCN	*M*/300 KCN	*M*/30 KCN	Uptake not inhibited by KCN (mm.³ per g. per hr.)
1	Ox kidney	320	42	52	50	160
2	Pig kidney	400	—	41	44	224
3	Sheep kidney	1700	75	80	82	310
4	Rabbit kidney	1100	57	79	79	230
*5	,,	560	65	73	72	150
6	,,	220	—	—	56	96
7	Pig liver	700	57	74	74	180
8	Sheep liver	360	43	62	61	140
9	,,	490	—	—	78	110
10	,,	660	—	—	83	110
11	,,	1800	—	—	91	160
12	,,	1500	—	—	89	160
13	,,	700	—	—	77	160
*14	,,	900	—	—	87	120
	,,	800	—	—	84	130
15	Rat liver	540	29	64	65	190
16	,,	860	—	—	65	300
*17	,,	490	33	51	59	200
*18	,,	760	48	69	74	200
*19	,,	680	—	—	66	230
*20	,,	690	4	42	69	210
*21	,,	600	—	42	—	—
*22	,,	500	—	48	—	—
*23	,,	600	—	—	61	233
	,,	580	—	—	56	255
*24	Rabbit liver	660	—	—	75	165
*25	,,	340	—	49	70	100
26	,,	580	—	—	76	140
27	,,	1200	—	—	88	140
*28	,,	1460	—	—	82	266
	,,	1800	—	—	89	195
*29	Ox liver	310	16	25	54	140
30	,,	360	25	—	—	—
31	,,	470	—	—	66	160
*32	,,	460	—	—	69	142
	,,	460	—	—	74	120

Table I (*continued*).

Exp. No.	Tissue	Rate of O_2 uptake without KCN (mm.³ per g. per hr.)	Inhibition produced by (in % of normal)			Uptake not inhibited by KCN (mm.³ per g. per hr.)
			M/3000 KCN	*M*/300 KCN	*M*/30 KCN	
33	Sheep heart	235	24	44	44	130
34	Ox heart	1450	36	71	83	245
35	Rabbit muscle	130	39	62	69	40
36	,,	220	—	—	74	56
37	Rat muscle	400	41	78	84	65
38	,,	270	—	—	63	100
*39	Fish muscle	20	30	50	50	10
40	Ox pancreas	90	33	40	25	54
*41	Sheep thymus	60	17	55	57	26
42	Ox spleen	300	0	0	33	—
43	,,	110	0	50	43	55
44	Pig spleen	170	0	62	53	65
45	Sheep spleen	100	0	55	—	—
46	Rabbit brain	700	40	76	91	65
47	Sheep brain, grey matter	600	—	—	83	100
	,, white matter	210	—	—	85	32
48	Ox brain, grey matter	300	—	—	73	80
	,, white matter	160	—	—	63	60
*49	Yeast	4000	—	—	85	600
*50	,,	2500	—	—	98	50
*51	,,	4500	—	—	94	290
*52	,, "fed"	36000	78	92	92	2800
*53	,, ,,	18000	44	83	83	3000
*54	,, ,,	41000	—	—	94	1500
*55	,, "starved"	1600	0	56	72	450

NOTES.

* All experiments were done at 40° except No. 39 (at 18°) and 50 (at 14°).

All experiments were done at p_H 7·3 except No. 51 (at 3·0).

All experiments were done on suspensions except Nos. 5, 17, 19–24 (on slices).

All experiments were done in buffer except Nos. 18, 20 (in Ringer).

All experiments were done on 0·5 g. tissue except Nos. 23, 28 (1st line with 0·3 g., 2nd line with 0·75 g.).

The respiration of the yeast was too rapid to permit of 0·5 g. being used, and the following amounts were taken:

Exps. 49, 51, 53, 55	0·1 g.
,, 50	0·2 g.
,, 52, 54	0·025 g.

In Exps. 5, 21, 22 the tissue slices were previously allowed to soak in the cyanide at 0° for 3 hrs., 20 mins. and 1·25 hrs. respectively.

In Exps. 25, 29, 41 the tissue had been kept some hours on ice. Exps. 14, 32 were done on the same samples fresh (1st line) and after keeping about 5 hrs. All the other tissues were absolutely fresh.

The yeast used was ordinary baker's yeast. "Fed" yeast denotes yeast which had been made up in 5 % suspension in 1 % glucose and incubated for 3 hrs. at 37°. Appropriate small amounts of such suspensions were then measured out into the respirometer flasks. "Starved" yeast denotes yeast which had been made up in 5 % suspension in buffer solution and aerated for 5 hours before taking samples.

The percentage inhibition is defined as $\frac{R_0 - R}{R_0} \times 100$, where R_0 and R are the rates of respiration in absence and presence of cyanide respectively.

The respiration is always given per g. fresh weight of tissue.

The values found for the respiration of the tissues in the absence of cyanide, given in the third column of Table I, are in good general agreement with those found by Barcroft and Shore [1912] by direct determination of the oxygen used by the organs *in situ* in the animal. For instance, they found

values for liver varying between 300 and 3000 mm.^{3} per hour per g. tissue (fresh weight), and for the viscera taken together they obtained values between 480 and 1080.

A number of points in Table I deserve special notice.

Excluding yeast, the part of the respiration which is sensitive to cyanide varies between about 50 % and 80 % of the whole. Only in rare cases does it reach 90 %.

The inhibition given by $M/300$ cyanide is usually the same as that given by $M/30$.

The first traces of the cyanide produce a large effect—in many cases $M/3000$ cyanide gives almost maximal inhibition. A striking exception to this statement is given by the spleen of all the species tested. In this case $M/3000$ cyanide never shows the slightest effect, and in one sample of spleen (Exp. 42) even $M/300$ did not affect the respiration rate. This strongly suggests that spleen, unlike all the other tissues tested, contains some substance which has a greater affinity for cyanide than the "respiratory enzyme," so that the first amounts of cyanide added are taken up by this, and are thus prevented from acting on the respiratory systems. Further work is, however, required on this point.

Another point of interest is that the cyanide-resistant part of the respiration fluctuates less than the total respiration. For instance, in the first 20 experiments the respiration varied between 200 and 2000 mm.^{3} per hour, while the cyanide-resistant respiration, given in the last column of the table, only varied between 100 and 300. It varies however from tissue to tissue, being low particularly in skeletal muscle and spleen (compare Exps. 2 and 37, for example). It may possibly be connected with some "endogenous" aspect of metabolism, not directly affected by nutritional conditions, etc.

In view of the importance of the results obtained in the above experiments, as limiting the extent to which Warburg's theory of cell respiration can be applied to animal tissues, it is desirable to consider what criticisms may be brought against the experiments, and what possible alternative interpretations of the results can be suggested.

There is first the possibility that the gas phase in the flasks contains HCN vapour given off from the solution at the beginning of the experiment, and that the apparent oxygen absorption in presence of cyanide is in reality due to the absorption of this HCN by the KOH used for absorbing the CO_2. This possibility is excluded in our experiments by the following considerations. (*a*) The same amounts of cyanide and KOH are present in both flasks of each Barcroft apparatus, and therefore such an uptake, even if it occurred, would not affect the readings of the manometer. (*b*) A control experiment was carried out without tissue, and with $M/30$ cyanide in the right-hand flask of the apparatus only, the left containing buffer solution. KOH on filter-paper was present as before. No movement of the manometer occurred, either in the direction of evolution or absorption. (*c*) The rate of uptake in

presence of cyanide is exactly proportional to the amount of any given tissue added; it was found that 0·6 g. of liver gave an absorption rate twice as great as 0·3 g. The rate is, however, different for different tissues. It is therefore clear that the effect is actually an uptake by the tissue.

The second possibility of error, arising out of the first, is that the cyanide has left the solution as HCN and been absorbed by the KOH in much the same way as the CO_2 produced by the tissue. This possibility is disposed of by the fact that at p_H 7·5 M/3000, M/300, M/30 and M/3 cyanide usually give the same degree of inhibition. The inhibitory effect of cyanide is reversible (see below), so that if the cyanide were being removed it should disappear from the M/3000 solution first and there should be no inhibition in this apparatus, and the inhibition should disappear from the others in turn. We have actually observed this effect in two experiments done in acid solutions. When it does take place, the indications are unmistakable. In these experiments the inhibition in the apparatus to which M/3000 cyanide was added was negligible. The uptakes with M/300 and M/30 both started at a rate corresponding to about 60 % inhibition, but while the one with M/30 remained at this rate that with M/300 gradually accelerated until its rate was the same as that without cyanide. These effects, which are clearly due to the removal of the cyanide from the solutions, only occur in acid solutions, and we have never observed such gradually increasing velocities at p_H 7·3, even with M/3000 cyanide. It may be mentioned that at the end of our experiments the odour of cyanide is detectable in the flasks with M/3000, strong in those with M/300, and very strong in those with M/30.

The next possibility is that the uptakes observed in presence of cyanide are due not to respiration but to oxidation of the cyanide itself. It has been stated by Ellinger [1924] that this reaction may occur in tissues under certain conditions. This, however, cannot be the case in our experiments, as the amount of cyanide present is quite insufficient to account for the uptakes observed. Consider for instance Exp. 3 in Table I. This experiment was continued for more than 2 hours, during which time the flask containing the tissue in M/300 cyanide absorbed over 340 mm.^{3} Even if the whole of the cyanide were oxidised the amount of oxygen taken up would only be 112 mm.^{3} Moreover, if the cyanide were being removed by such oxidation, the inhibition would tend to disappear, and, as has just been mentioned, this is never observed. In Exp. 3 the rates, even with only M/3000 cyanide, remained unchanged after 2 hours. In a few experiments, however, such as Nos. 40, 43, 44 in Table I, we found that the oxygen uptake was slightly greater in M/30 cyanide than in M/300. We are inclined to attribute this effect to a slight oxidation of the cyanide. With the majority of tissues however it does not occur.

The form of the curves disposes also of the objection that the cyanide may not penetrate into the tissue sufficiently to exert its full effect. For the first traces of cyanide exert an immediate marked action, and the degree of

the inhibition produced does not increase with time. In order to make quite certain, however, a few experiments were carried out in which the tissue slices were allowed to soak in the cyanide solutions for some time before measuring the respiration rate. These are indicated in Table I, which shows that no effect was observed.

The possibility that the cyanide does not inhibit more completely because it is preferentially combined with substances other than the "respiratory enzyme," or because the complex which it forms with the latter is partially dissociated, is again negatived by the fact that the first traces usually produce the maximum inhibition and a further increase in concentration of a hundred times does not increase the inhibition. Such effects, however, are probably playing a part in those cases where maximal inhibition is not reached with a concentration of $M/300$.

A more serious criticism, if justified, is that our measurements of the respiration rate in the absence of cyanide, since this is much higher than that in the presence of cyanide, may in fact have been limited by the rate at which oxygen could diffuse from the gas into the liquid, so that the true respiration rate, and therefore the percentage inhibitions, are in reality greater than those given. The question of diffusion effects will be fully dealt with in a forthcoming paper, where it is shown that the apparatus is capable of dealing with much more rapid uptakes without errors due to this cause. We have, however, carried out one or two further experiments with animal tissues which definitely disprove this criticism in the case of the experiments given above. In the first place we have found that increasing the amount of tissue taken does not affect the degree of inhibition produced by $M/30$ cyanide, as shown in Exps. 23 and 28 in Table I[1]. This result would not have been obtained had the rate of uptake been limited by the apparatus; for increasing the amount of tissue would not increase the normal uptake if this were limited by diffusion, while the uptake with cyanide, being much smaller, would not be limited thus and would increase with the amount of tissue. Thus, in such a case the more tissue taken the less would be the degree of inhibition observed. This is not the case.

Another experiment, represented in Fig. 7, was carried out by varying the amount of tissue taken and measuring the oxygen uptake in the absence of cyanide. The results showed that the rate of uptake was exactly proportional to the amount of tissue taken, so that diffusion effects could certainly be neglected, provided the rate was not much greater than the maximum rate represented on the curve, *i.e.* about 800 mm.^{3} per hour. That is to say, in Table I, where 0·5 g. of tissue was normally employed, the figures give true values in all cases where the uptake does not exceed 1600 mm.^{3} per g. per hour. As a matter of fact the maximum rate is probably much greater than this. We found also that both the rate of shaking and the partial pressure

[1] The slight differences are within the experimental error, and, in the case of the more rapid uptake, in the opposite direction.

of oxygen in the apparatus could be widely varied without affecting either the respiration rate or the degree of inhibition produced by cyanide (see the results in Table II).

Finally it may be objected that the tissue is not under normal conditions in the experiments. To this we will only say that a slice of perfectly fresh tissue, cut with a razor moistened with Ringer's solution, and suspended in

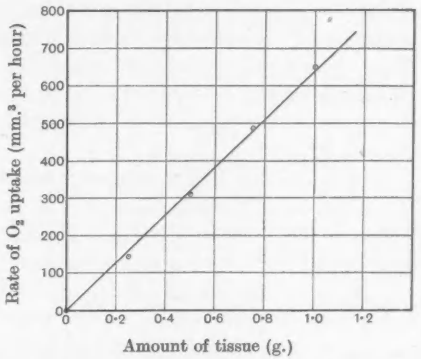

Fig. 7. Observed rates of oxygen uptake with varying amounts of rat liver in the absence of cyanide.

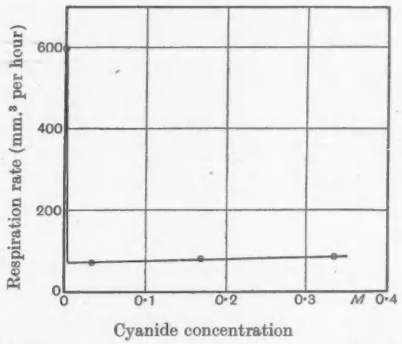

Fig. 8. Effect of higher concentrations of cyanide on respiration of rabbit liver.

this solution with free access of oxygen, is probably as close an approximation to the normal tissue as it is possible to get in experiments of this type; and the slight variations from these conditions which we have made have been shown to have no effect on the results.

A few other experiments on the effect of cyanide remain to be considered. Fig. 8 shows an experiment in which the cyanide solutions were 10 times the normal strength. Even with $M/3$ cyanide no greater inhibition is produced.

Fig. 9 shows that the cyanide effect is reversible: on removing the cyanide by washing, the inhibition disappears and the respiration returns to its former rate. The reversibility of cyanide inhibition is of course well known.

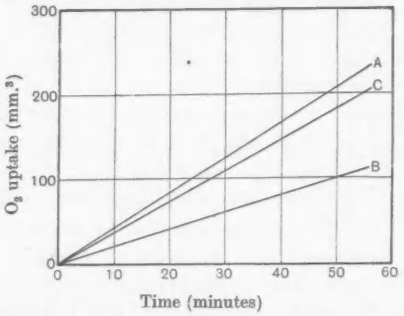

Fig. 9. Reversibility of cyanide inhibition. Thin slices of rat liver were cut with a razor moistened with Ringer. Three portions of 0·5 g. were taken. *A* was placed in 2·5 cc. buffer solution, *B* and *C* in 2·5 cc. buffer containing *M*/30 cyanide, and all kept at 0° for 1 hour. Then the slices were taken out, quickly rinsed with buffer solution, and lightly dried with filter-paper. *A* and *C* were then immediately placed in 2·5 cc. fresh buffer in respirometers, *B* in 2·5 cc. buffer containing *M*/30 cyanide, and the oxygen uptakes measured.

The effect of increasing and diminishing the partial pressure of oxygen in the flasks is shown in Table II. The flasks were filled with mixtures of oxygen and nitrogen in different proportions, the percentages of oxygen being as indicated in the third column of the table.

Table II. *Effect of oxygen pressure.*

Exp. No.	Tissue	O_2 %	Rate of oxygen uptake (in mm.^{3} per g. per hr.) in presence of: No KCN	*M*/3000 KCN	*M*/30 KCN	Inhibition %
1	Rabbit liver	20	620	—	170	72
	,,	100	600	—	180	60
2	Sheep liver	20	490	—	110	77
	,,	100	500	—	120	76
3	,,	7	660	—	125	81
	,,	20	660	—	140	79
4	Ox liver	7	340	270	—	21
	,,	20	350	270	—	23

As the figures show, wide variations of oxygen pressure in both directions produced no alteration in either rate of oxygen uptake or degree of inhibition. Exp. 4, in which only enough cyanide was added to produce a rather slight inhibition, shows that there is no competition between the oxygen and the cyanide.

A few determinations of the effect of cyanide on the respiratory quotient were made by the following method. Four respirometers were used with the same amount of tissue in all. One pair of these had KOH on filter-paper to absorb the CO_2; in the other pair this was omitted. To one of each pair *M*/30 KCN was added, the other two being without cyanide. The pair without

KOH were provided with small tubes which could be upset into the solution. These contained strong oxalic acid, and were upset at the end of the experiment in order to drive off CO_2 from the buffer solution. The reading of the first pair of respirometers at the end of the experimental period gave of course the oxygen used with and without cyanide. The differences between the readings of the corresponding members of the first and second pair gave the amount of CO_2 evolved in the presence and absence of cyanide. From this, however, the amount of CO_2 already present in the tissue and buffer at the beginning of the experimental period had to be subtracted. This was determined by setting up a third pair of respirometers, exact duplicates of the second pair, in which the oxalic acid was upset at the beginning of the experiment. The amount of CO_2 thus liberated was then subtracted from the gross amount given by the second pair, to give the amount actually produced during the experimental period. Using this technique it was found that the respiratory quotient was always lowered by the addition of cyanide. For instance, the value found for slices of rat liver in the absence of cyanide was 0·8, and with $M/30$ KCN this fell to 0·64.

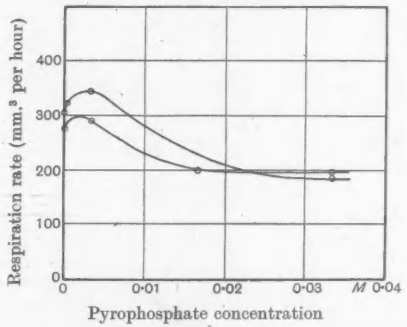

Fig. 10. Effect of pyrophosphate on respiration of two different samples of rat liver.

In addition to the experiments described above on the effect of cyanide on cell respiration, experiments were also performed using pyrophosphate instead of cyanide. Pyrophosphate acts as an inhibitor in many iron-catalysed reactions, but according to Keilin [1929] does not inhibit the "respiratory enzyme." In all experiments the pyrophosphate was freshly made up and adjusted to p_H 7·3 before use. In Fig. 10 are given two inhibition curves showing the effect of neutral pyrophosphate on rat liver. The inhibition is much less than with cyanide, as was expected, and a much higher concentration is required before any inhibition appears. These results are typical of liver. Muscle, however, behaves differently in that pyrophosphate is actually considerably stronger than cyanide as an inhibitor, as shown in Fig. 11. With yeast pyrophosphate has not the slightest effect. It may, however, be unable to pass through the cell membrane into the interior of the yeast cells.

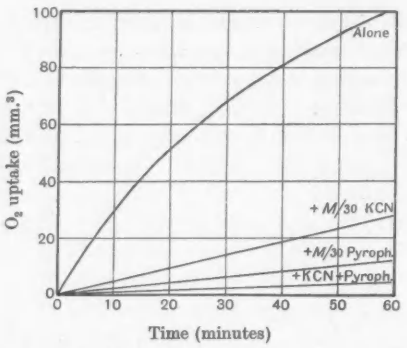

Fig. 11. Effect of pyrophosphate and cyanide on respiration of rabbit muscle.

In Table III are summarised the results of a number of comparative experiments showing the relative effects of $M/30$ pyrophosphate and $M/30$ cyanide on the respiration of a number of tissues. The effect of adding pyrophosphate and cyanide simultaneously is also shown.

Table III.

Exp. No.	Tissue	Rate of O_2 uptake by tissue alone (mm.³ per g. per hr.)	Percentage inhibition with		
			$M/30$ Pyroph.	$M/30$ KCN	$M/30$ Pyroph. + $M/30$ KCN
1	Rat liver	600	40	—	—
2	,,	560	30	—	—
3	Ox liver	470	28	66	74
4	,, fresh	460	30	69	80
	,, kept	460	30	74	85
5	Sheep liver	1800	75	91	95
6	,, fresh	900	64	87	91
	,, kept	800	60	84	90
7	,,	1500	70	89	93
8	Rabbit muscle	220	87	74	95
9	Rat muscle	270	67	63	82
10	Yeast	4000	0	85	85
11	,,	4500	0	94	93

In order to determine whether the brief delay, unavoidable in some cases, between the death of the animal and the beginning of the respiration measurements had any effect, several comparative experiments were made between absolutely fresh tissues and portions of the same tissues which had been kept at room temperature for some hours before slicing or mincing. Fig. 12 and Exps. 4 and 6 of Table III are such experiments, and they show that the effects of a short delay can be neglected.

The inhibiting effect of pyrophosphate, like that of cyanide, is reversible, as the experiment represented in Fig. 13 shows.

The results given in Table III seem to give indications of some degree of parallelism between the cyanide and pyrophosphate inhibitions, at any rate with a given kind of tissue. For instance, in the seven samples of liver for

which both the inhibitions are given, the order of sensitivity of the samples is the same for pyrophosphate as for cyanide. It is not clear why this should be so, since it is commonly assumed that the effect of cyanide is due to its action on the "respiratory enzyme" (indophenol oxidase), whereas pyrophosphate has no action on this, so that they would be acting on quite different

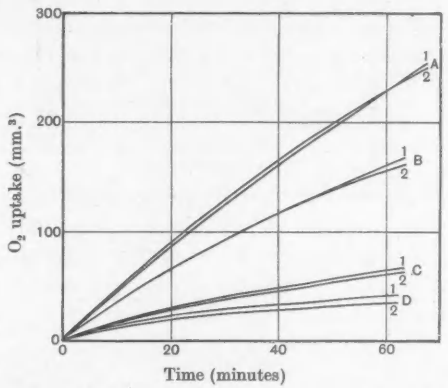

Fig. 12. Effect of pyrophosphate and cyanide on ox liver, fresh and after keeping 5 hours. *A*, tissue alone; *B*, tissue + *M*/30 pyrophosphate; *C*, tissue + *M*/30 cyanide; *D*, tissue + *M*/30 pyrophosphate + *M*/30 cyanide. Curves numbered 1 on fresh tissue, those numbered 2 on kept tissue.

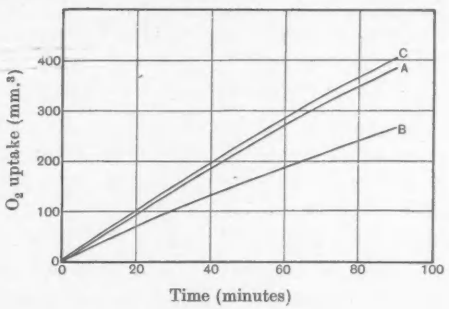

Fig. 13. Reversibility of pyrophosphate inhibition. The experiment was done on rat liver slices in exactly the same way as that given in Fig. 9, using *M*/30 pyrophosphate instead of *M*/30 cyanide.

systems. It has not, however, been definitely established that the inhibiting action of cyanide on respiration is entirely due to its action on the "respiratory enzyme," for it is known to inhibit other oxidation catalysts in the cell, and it is not unlikely that some of the systems inhibited by pyrophosphate will also be inhibited by cyanide.

It is, however, certain that pyrophosphate acts upon certain systems which are not poisoned by cyanide, at any rate in muscle, where the inhibition it

produces is greater than the maximum cyanide inhibition. One such system is the succinodehydrase, as shown in the experiment represented in Fig. 14. This is an unpublished experiment carried out by Dixon and Thurlow in 1925. While $M/1000$ pyrophosphate had a definite inhibiting action on the reduction of methylene blue by this system, it was found that cyanide, even up to $M/50$, had absolutely no effect on the reaction. On the other hand, even $M/25$ pyrophosphate had no effect on the reduction of methylene blue by the xanthine oxidase system.

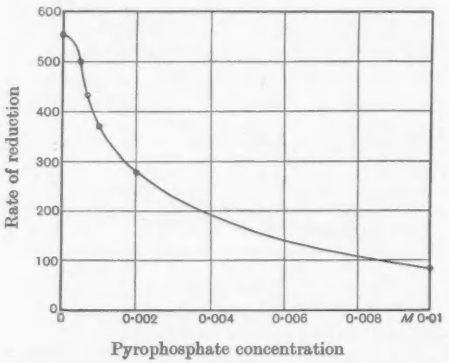

Fig. 14. Effect of pyrophosphate on rate of reduction of methylene blue by succinoxidase. Each Thunberg tube contained 2 cc. succinoxidase solution + 0·5 cc. 1/5000 methylene blue + 0·2 cc. (= 0·4 mg.) succinate + 1·3 cc. phosphate buffer p_H 7·6 containing the necessary amount of pyrophosphate. "Rate of reduction" = 10,000 × reciprocal of reduction-time in minutes.

Discussion.

We consider that the experimental evidence presented in this paper shows quite definitely that the respiration of typical animal tissues is made up of two parts, one of which is inhibited by cyanide while the other is cyanide-stable; and that, while the relative proportion of the two parts varies in different tissues, the latter usually contributes a considerable part, sometimes more than half, to the total respiration. Thus, a considerable part of the respiration must be due to systems which are not poisoned by cyanide. As all Warburg's mechanisms are cyanide-sensitive, his theory can no longer be regarded as able to account for the whole of the respiration of animal tissues; and it can no longer be maintained that all oxidations by molecular oxygen in the cell are brought about by the same type of mechanism.

In the light of this evidence Warburg's remarks quoted in the introduction to this paper are seen to lose much of their force; and in particular this applies to his argument that those tissue oxidases which are unaffected by cyanide cannot be regarded as respiration systems because they behave unlike respiration, since it is now clear that only one part of the respiration behaves in the manner referred to. Such cyanide-stable systems as the xanthine

oxidase may well contribute to the cyanide-stable part of respiration. Of course the xanthine oxidase itself cannot account for the whole of this part (it is altogether absent from muscle [Morgan, 1926]), and it seems likely that there are a number of other systems of this type.

The recent important work of Keilin [1929] has shown that the "respiratory enzyme" is to be identified with the well-known indophenol oxidase of tissues. Cyanide, CO, etc., act upon this, and the inhibition of respiration is due, in part at any rate, to the poisoning of this enzyme. It acts by oxidising cytochrome, which is in turn reduced by the dehydrase systems. Our experiments show that the indophenol-oxidase-cytochrome mechanism likewise cannot account for the whole respiration of animal tissues. They do not of course show how much of the total respiration is actually due to this mechanism, they merely set an upper limit to its action; for it is not yet established that this system is responsible for the whole of the cyanide-sensitive respiration of animal tissues, and the cyanide may act upon other systems as well.

Summary.

1. A study has been made of the effect of various concentrations of cyanide on the rate of respiration of a number of animal tissues.

2. In most cases a cyanide concentration of $M/1000$ is sufficient to produce the maximum degree of inhibition obtainable. Above this point no greater inhibition is produced by increasing the cyanide concentration 100 times.

3. The maximum inhibition varies in different tissues between 40 % and 90 %, the average value being about 60 %.

4. It is concluded that the respiration of animal tissues is made up of two parts. One, accounting for about two-thirds of the total, is due to systems poisoned by cyanide; the other one-third is due to systems which are stable to cyanide.

5. Warburg's "respiratory enzyme" (Keilin's cytochrome-indophenol-oxidase system) can therefore only account at most for about two-thirds of the total respiration of animal tissues.

6. The respiration of spleen, unlike that of all the other tissues tested, is not affected by small amounts ($M/1000$) of cyanide. It appears to contain some substance with a greater affinity for cyanide than the "respiratory enzyme."

7. Negelein's statement that the respiration of yeast is almost completely inhibited by cyanide is confirmed. Some samples of yeast, however, show an incomplete (85 %) inhibition.

8. The effect of pyrophosphate on the respiration rate of liver, muscle and yeast has also been studied. With liver, small concentrations produce a slight acceleration, but larger amounts produce inhibitions which vary between 30 % and 70 % but are always considerably less than the corresponding

cyanide inhibitions. With muscle, on the other hand, pyrophosphate produces a greater inhibition than cyanide. Pyrophosphate produces no effect on yeast: possibly the cell-membranes are not permeable to it.

9. Since the "respiratory enzyme" is not inhibited by pyrophosphate, the inhibition of respiration must be due to its action on some other system. It is shown that pyrophosphate inhibits the reduction of methylene blue by the succinoxidase system, though this reaction is unaffected by cyanide.

10. The actions of both cyanide and pyrophosphate on respiration are reversible.

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